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Electrophoresis.

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THEORY, METHODS, AND APPLICATIONS

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Edited by

MILAN BIER

Fordham University, New York
and Institute of Applied Biology, Inc., New York

1959

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
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ACADEMIC PRESS INC.
111 Fifth Avenue
New York 3, N. Y.

4166
E3,65 N59

Library of Congress Catalog Card Number 59-7676

CFTRI-MYSORE
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Electrophoresis..

PRINTED IN THE UNITED STATES OF AMERICA

DEDICATION

THIS VOLUME is dedicated to the individual research worker, whose contributions, often unheralded or unrecognized, make books such as this possible.

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PREFACE

The contribution of electrophoresis to our knowledge of proteins is second to no other method. Its impact is felt in biochemistry, physiology, and medicine.

Recent years have witnessed a broad development of new experimental techniques and their application to a variety of other substances. It appeared therefore, that an authoritative presentation, dealing with the theory, methods, and applications in their proper perspective would fill a gap in the existing literature. It is to be hoped that this has been accomplished in the present collaborative endeavor, and that the collected essays will be of value not only to experimental and theoretical workers in the field but also to all researchers concerned with broader aspects of proteins, polyelectrolytes, and colloids in general.

The emphasis in the exposition is on the fundamental principles involved in electrophoresis, the problems encountered therein, and the means of solving them. Detailed laboratory procedures are included inasmuch as they serve to illustrate these problems. All major areas of application were covered, encompassing clinical medicine, studies of proteins, viruses, bacteria, and other biological materials, analytical use in organic and inorganic chemistry, and preparative and industrial uses.

The editor takes pleasure in thanking all contributors for their arduous collaboration, and in acknowledging the advice especially of Dr. L. G. Longsworth in the preparation of the book.

M. Bier

New York City
December, 1958

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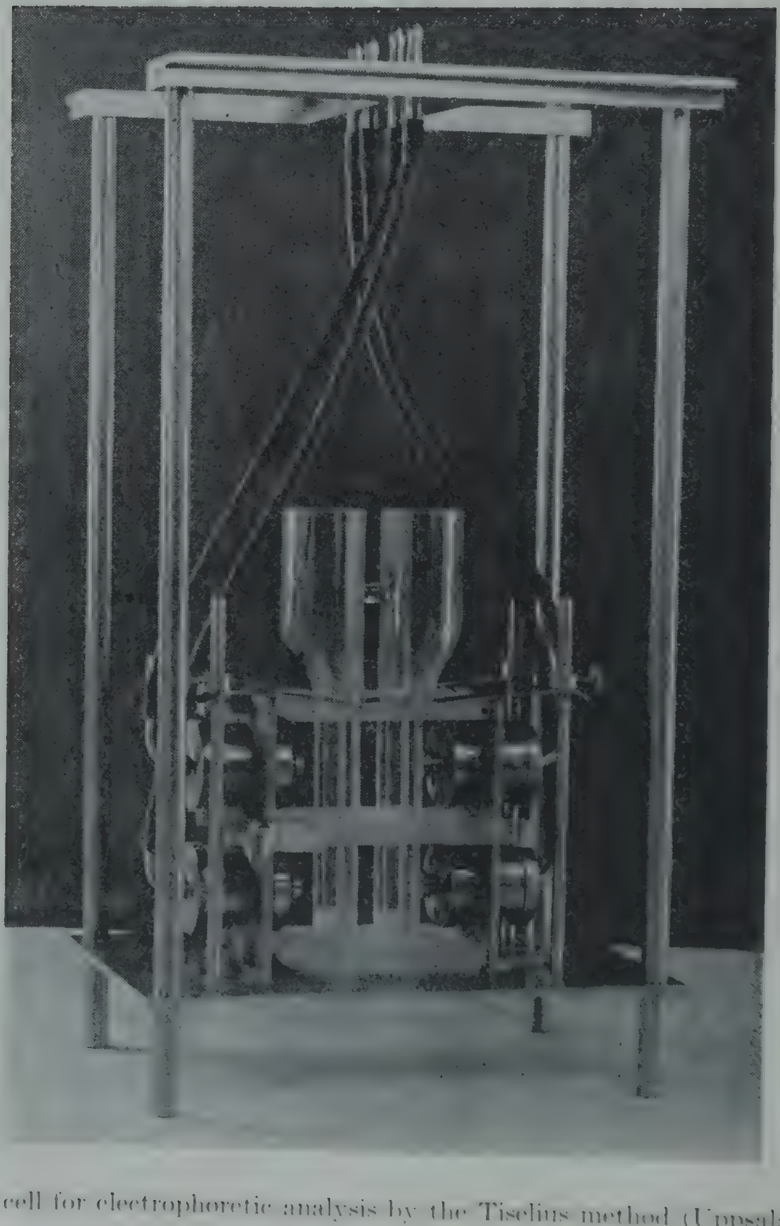

The first cell for electrophoretic analysis by the Tiselius method (Uppsala, 1937)

Introduction

ARNE TISELIUS

The term "electrophoresis" or "cataphoresis" was originally introduced to mean the migration of charged colloidal particles in an electric field. Just as the definition of a colloid, and particularly the distinction between colloidal and molecular systems, necessarily is rather vague, there has been some diversity of opinion about how wide use the term "electrophoresis" should have. This has become particularly evident in the many applications in chemistry (particularly biochemistry) of the migration of substances in an electric field. Some authors apparently prefer the term "ionophoresis" for substances of lower molecular weight, particularly when the experiments are made in stabilized media such as gels and powders. This term has even sometimes been used to distinguish such stabilized migration from migration experiments in free solutions, which does not appear logical.

During the last decade there has been a particularly rapid development in the application of methods of this type, covering the whole field from the largest protein molecules down to, for example, amino acids, sugars, purines, pyrimidines, and even simple inorganic ions, using essentially the same types of apparatus and procedures, irrespective of the molecular size of the substances studied. For this reason—and possibly also for historical reasons—most research workers in the field seem to prefer the well-established term "electrophoresis" as a common name for all these procedures, especially if separation problems are the chief purpose of the investigation. The situation is somewhat analogous to the use of chromatography, which nowadays is certainly not limited to colored substances.

In the physicochemical theory of the migration of small ions there is, according to Debye-Hückel, an "electrophoretic" factor which depends upon the diffuse double layer in the ionic atmosphere surrounding each ion. The role of this factor becomes the more pronounced, the larger the size of the ion and the higher the ionic strength of the solution. There is thus a continuous transition from free ionic migration to electrophoretic phenomena, where surface potentials and double layer dimensions are decisive.

From the very beginning of electrophoretic studies on colloids, the relationship between the mobility and the surface charge or surface potential came into the foreground. The electrophoretic migration could be studied with relatively simple means and gave a convenient way of determining these properties, which are of fundamental importance for the stability of many colloidal systems, especially those of a hydrophobic type. The early investigations by Picton and Linder (1, 2), Hardy (3, 4, 5), Ellis (6), and Powis (7) give examples of this type of work. The discovery by Hardy that many biocolloids, like proteins, enzymes, etc., show characteristic electrophoretic mobilities, which largely depend upon the pH of the solution, greatly stimulated the interest in electrophoretic work (3, 4, 5). Now the *characterization* of such substances by means of their electrophoretic properties, and particularly of their isoelectric points, began to attract the interest.

An early example is the classical work of Michaelis (8), who determined the isoelectric points of enzymes by migration experiments at different pH's. It is interesting that this characterization could be done before pure enzymes were available: Michaelis determined the direction of the migration in a U-tube by analyzing the two limbs of the tube for enzymatic activity after current had passed through.

At that time very little was known about the chemical nature and physical properties of proteins and enzymes. It is only natural that their electrophoretic properties aroused interest, since these properties appeared to be to a certain extent characteristic and lent themselves to quantitative studies, even though accurate measurements proved to be difficult to make.

Since then electrophoretic methods have been successively refined. The conditions for disturbance-free migration have been established, and accurate quantitative measurements of migration velocities in different media have been made for a large number of biologically important substances, particularly for proteins. One important method depends upon the study of the migration of the boundary of a protein dissolved in a buffer solution of accurately defined pH and layered below a buffer solution of the same composition (the "moving boundary method"). The introduction of highly sensitive optical methods for observing the migration of the boundaries and of their resolution into several components in complex systems not only facilitated accurate mobility measurements but also opened up possibilities for an "electrophoretic analysis" of mixtures. Such systems are very frequently encountered in biochemical work, where the substances are obtained from extracts of biological material. The improved methods gradually led to results which demonstrated the high degree of specificity of the electrophoretic properties. Substances which had been believed to be reasonably well defined by common criteria, e.g. crystallization, sometimes were found to yield two or more components in electrophoretic analysis, and the method has now found wide use as one important criterium of purity for substances which are otherwise difficult to define.

The information derived from electrophoretic investigations has been considered particularly reliable in such cases on account of the gentleness of the method. The application of other separation procedures, involving precipitation or the use of nonaqueous solvents or other aggressive chemicals, may easily cause damage to substances as unstable as are many biocolloids. Besides this, coprecipitation and adsorption phenomena are apt to interfere in fractionation work by such methods when one deals with crystals or precipitates of substances of large molecular weight. In electrophoretic separation (as in ultracentrifugation and similar "transport methods") the migrating substances remain in the same medium during the entire process and the observation method itself is apt to give warning of eventual irreversible changes accompanying the separation.

The diversity of electrophoretic behavior among biochemically important substances is very gratifying in relation to separation. It is evidently unusual for two different proteins to show the same mobility over the entire pH range accessible for investigation, and even small differences in migration may be utilized with the modern methods of investigation.

Thus the *separation* aspect has come into the foreground to an increasing extent. Electrophoretic analysis by the moving boundary methods, using refractive index observation, gives a high resolution and is suitable for characterization of the observed components by their mobilities, which can be measured under well-defined conditions. The method gives, however, only a boundary separation, and at the end of an experiment the components are only partially separated. Only some of the fastest and slowest components can be taken out of the electrophoresis tube. Thus, this type of electrophoretic analysis serves the investigator more as a guide for separation by other procedures than as a separation method in itself. To a certain extent there is thus an analogy to the use of spectral analysis in the purification of metals.

The problem of a real electrophoretic separation "in substance" thus became an important issue. It appeared very tempting to utilize the efficient separation, which can be observed optically in the moving boundary methods, in order to isolate the components for further investigation of their chemical and biochemical properties and eventually also to work out methods suitable on a preparative scale. The overlapping "boundary separation" had thus to be substituted by a "zone separation" in which each component was allowed to form a zone separated from the others by empty regions. This, however, necessitated a radical change in the method. Zones of this kind are gravitationally unstable and would give rise to convections in the ordinary electrophoresis tube. To avoid this, the tube must be packed with a stabilizing medium, like starch, cellulose, or glass powder, or the experiment must be run in a gel or in a strip of filter paper.

However, it was not only the need for complete separations which gave rise to this development. Several successful early attempts had been made to run electrophoresis experiments in gels or strips of filter paper, for which only a very simple apparatus is needed and very small samples of the material to be investigated are required. The development of paper strip electrophoresis obviously received a great stimulus from the great success of paper chromatography and from the increasing need for a simple and rapid micromethod for electrophoretic analysis of sera in the clinic.

We have in Uppsala suggested the name "zone electrophoresis" for methods of this type, to distinguish them from "boundary electrophoresis" of the kind referred to above. Zone electrophoresis may be performed in stabilizing media of different kinds (or in density gradients) in columns or troughs of various types, and there are also constructions allowing continuous separation of large quantities. Paper electrophoresis is related to these methods very much in the same way as paper chromatography to column chromatography.

Experience has shown that the zone methods can be applied also to low molecular weight substances, like peptides, amino acids, and nucleotides, which cannot easily be studied by boundary electrophoresis. Thus a considerable extension of the use of electrophoretic analysis has taken place, and the method often can compete with chromatography, for example in the fractionation of protein hydrolyzates. It cannot be helped, however, that in introducing carrier systems, necessary for the stabilization of the zones in zone electrophoresis, some of the possibilities of quantitative mobility determinations have to be sacrificed. The stabilizing material is present in an enormous excess over the amount of substances studied, and interactions influencing mobilities in an uncontrollable way can hardly be avoided, even though attempts are being made to find as inert filling materials as possible. Paper electrophoresis experiments are also subject to other complications, e.g. by evaporation from the strip, which is heated by the current passing through. In such cases one has to refer the observed migration to that of known substances in parallel experiments, run simultaneously.

It is only natural that if the purely preparative or separation aspects dominate the purpose of the experiment one is ready to make a sacrifice of some of the quantitative interpretations, especially if very high resolution can be obtained. However, the possibility of identification of new components, visible as boundaries or zones, by their mobilities has always been an attractive feature of the electrophoretic methods. It is therefore to be hoped that such procedures, where other phenomena interfere—and perhaps even contribute to the separation efficiency—can also be understood in all their details so that a rational prediction of separation efficiency in such systems can be made.

Already in the early history of electrophoresis, attempts were made to construct separation apparatus of considerable capacity. Multicompartment apparatus with membranes have been used with success, and also other types, where the migration is followed by suitable analytical methods. Another type of procedure—electrophoresis convection—has been worked out by Kirkwood and his collaborators and appears to be very efficient for the preparative separation of larger quantities of material.

The microscopic method, where the migration of colloidal particles is observed directly with a microscope, has played an important role in the study of the fundamental physicochemical laws governing electrophoretic phenomena. This method has possibilities also in the characterization of biological materials, and there are some very interesting recent results by this method in the characterization of bacterial surfaces.

This introduction has dealt chiefly with the application of electrophoresis to biochemical substances in aqueous solutions, usually in buffered media of defined pH, and there is no doubt that such work dominates the field today. One should not forget, however, the possibilities of influencing the electrophoretic properties by other components in the medium, for example ions other than H^+ and different complex-forming agents. The separation of sugars and polysaccharides in media containing borate forms a beautiful illustration of the application of this principle.

Electrophoresis in nonaqueous solvents has been explored very little. Naturally the electrical conductivity in such media is governed by laws different from those valid in aqueous solutions, and the conductance often is very low. This, on the other hand, may be compensated for by the possibility of applying high voltage without incurring disturbingly large heating effects as the current will be low. Studies of such systems would no doubt be well worth undertaking.

The development of electrophoretic methods and their application form an unusually beautiful example of the fruitfulness in the application of physical methods to biochemical and biological problems. It is to be hoped that future development—and much remains to be done—will benefit from a similar intimate collaboration between representatives of different fields of science.

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Chapter 1

Electric Potentials in Colloidal Systems

J. TH. G. OVERBEEK and J. LIJKLEMA

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I. Introduction

Electrophoresis is the movement of charged particles suspended in a liquid, under the influence of an applied electric field. The usual aims of electrophoresis experiments are the obtaining of information on the electrical double layers surrounding the particles, the analysis of a mixture, or its separation into components.

The first aim requires a theory connecting the electrophoretic velocity with fundamental quantities related to the double layer, such as electric potential, charge, and structure. It is the purpose of this chapter to treat the meaning of the notions charge and potential of the double layer, to show clearly where confusion with relation to these concepts might arise and to treat their interrelation with electrophoresis and other electrokineti phenomena.

The theoretical treatment of the electrical double layer depends on its geometry. The most simple case, *viz.*, the double layer at a flat interfac will be treated first. With this example, already many facts connected with double layers can be explained. Later in this chapter attention will be paid to more complicated systems: spherical particles, polyelectrolytes, and proteins.

II. General Considerations on the Structure of the Boundary Layer

The boundary between two phases is not a mathematical plane, but a layer of finite dimensions. The properties of the two adjacent phases change gradually over a certain distance. These changes depend both on the forces between molecules (van der Waals' forces, purely electrostatic ones, hydrogen bonds, etc.) and on geometrical factors (fitting of the two surface layers onto one another). Even in a one-component system the density and orientation of the molecules change gradually when going, for example, from the liquid to the gas phase. This phenomenon has been treated extensively by Bakker (1) in his theory of capillarity. In a multicomponent system the concentrations in the boundary layer are as a rule different from those in bulk, thus leading to what is commonly called *adsorption*.

In most cases the changes near the phase boundary are limited to a very few layers of molecules, but there all properties of the phases are changed, including structure, density, composition, dielectric constant, viscosity, etc. If one or both phases contain ions, the transition layer may be much more extended. In this case it frequently occurs that one type of ion is strongly concentrated at the phase boundary by short-range forces. Ions with a sizable hydrophobic part, like those of detergents, although reasonably soluble in water, will show a tendency to be expelled from the aqueous phase into its boundaries with any less polar phase and so accumulate especially at air-water or oil-water interfaces. Similarly Ba^{++} and SO_4^{--} ions will be more readily adsorbed by $BaSO_4$ crystals than most other ions because they fit into the $BaSO_4$ lattice. Many other examples could be given.

But if ions of one sign are adsorbed at the phase boundary, ions of the opposite sign will be attracted by the resulting electric field and accumulate also near the phase boundary. This accumulation of "counterions" (*Gegenions*), however, will be counteracted by their Brownian motion. The result is the formation of an electrical double layer electrically neutral as a whole which may extend over a thickness of several hundred Ångströms or even more. The structure of this double layer will be treated more completely in the following sections.

Electrophoresis involves a tangential motion of one phase with respect to the other. It occurs only if the two phases carry free charges of opposite sign. Orientation of dipole molecules near the phase boundary, although creating a potential difference between the two phases, cannot give rise to electrophoresis because the charges of the dipole cannot be separated permanently by the applied electric field! So electrophoresis is intimately connected with the ionic part of the electrical double layer and any theoretical approach to electrophoresis has to start with a study of this electrical double layer.

III. Charge and Potential in the Electrical Double Layer

A. Charge

As mentioned above, the total charge of the electrical double layer is zero, but in it ions and electrons may be accumulated preferentially by adsorption. Analytical measurements allow in principle, although not always in practice, the determination of the amount of ions of any kind adsorbed at the phase boundary. Such measurements, however, do not yield any information on the distribution in space of these charge carriers. But quite often relatively simple theoretical considerations come to our aid. If it is known that, for example, electrons and sodium ions are accumulated at the phase boundary between a metal and a salt solution, it is obvious that the electrons are present in the metal phase and the sodium ions in the aqueous phase. We thus consider the metal to be negatively charged with respect to the water phase, the amount of charge being given by the charge of the excess electrons or by the charge of the sodium ions with sign reversed. Further information on the spatial distribution of the charge carriers demands more refined theories such as will be treated in Section IV, Ionic Double Layer.

B. Potential

The spatial distribution of electric charge is of course connected with an electric potential. The exact nature of electric potentials in and near phase boundaries is, however, a subject with numerous pitfalls. Considering the potential difference between two points in space as defined by the amount of work to be done in transferring a given charge from one point to the other, the question immediately presents itself whether this amount of work is independent of the material carrying this charge. We may expect to find the same amount of work involved in the transport of a hydrogen ion from point A to point B in an aqueous solution as in the transport of a sodium ion between these same points. But, as soon as a phase boundary is crossed these amounts of work are generally different because, apart from the purely electrostatic work, configurational work also called chemical work, is performed in changing the immediate surroundings of the sodium (or hydrogen) ion. This configurational work differs for H^+ and Na^+. Moreover, there are no means of separating the chemical and the electrical work in an unequivocal way, and, as there is no good reason to prefer one charge carrier above the other, it seems as if we do not have any way to determine the electrical work and thus the potential difference between two different phases. The same difficulty, although perhaps to a lesser extent, is valid for the determination of the potential difference between two solutions of different composition.

C. Electrochemical Potential

Guggenheim (2) has repeatedly pointed out the impossibility of separating the chemical and the electrical parts in the work of transporting ions of species i and has introduced the notion of *electrochemical potential*, η_i, composed of the *chemical potential*, μ_i, and a term $z_ie\varphi$, where z_i is the charge of the ion and φ the electric potential.

$$\eta_i = \mu_i + z_ie\varphi \tag{1}$$

In any actual transport process only electrically neutral combinations can be transported. In that case the terms $z_ie\varphi$ add up to zero, and for *electroneutral combinations*

$$\sum_i \eta_i = \sum_i \mu_i \tag{2}$$

Still it is customary to use the notion "potential difference between two phases," and in order to see clearly what this may mean, we shall treat briefly the galvanic cell.

D. Galvanic Cells

The electromotive force (e.m.f.) of a galvanic cell is theoretically well defined; it is closely connected with the total chemical process in the cell [see, e.g. (3)], and measurable in an unequivocal way as the potential difference between the leads connected with the electrodes. These leads are of the same material, and the potential difference measured between them is thus independent of the charge carrier used.

On the other hand, the e.m.f. can be considered as the sum of the potential jumps occurring at each of the phase boundaries in the cell. Although, as shown above, each of these potential jumps separately is inaccessible to measurement, their sum is well defined. It is often possible to vary one of the phases in the cell, e.g. by changing the concentrations in a solution, in such a way that of the potential jumps at the phase boundaries involved only one is expected to be seriously affected. In such a case the change in e.m.f. corresponds directly to a change in the potential jump at one single phase boundary.

The cell used for measuring the pH may serve as an example. This cell, which contains five phase boundaries, can be described as follows:

Pt	|	Solution A containing H$^+$ ions and saturated with H$_2$ at 1 atm.	⋮	Saturated KCl	⋮	KCl, 0.1 M saturated with Hg$_2$Cl$_2$	|	Hg	|	Pt
	1		2		3		4		5	

If concentrations in the solution A are varied, only the phase boundaries *1* and *2* are affected. If solution A contains a relatively large amount of a neutral electrolyte, e.g. Na$_2$SO$_4$, and a small concentration of a buffer system, variations in the buffer system will practically not affect the diffusion potential at the liquid junction *2*, but they will affect the potential jump between platinum (Pt) and the solution, and this last change is reflected in the change of the e.m.f. of the cell. Written as an equation:

$$\Delta(\text{e.m.f.}) = \Delta(\varphi_{\text{solution}} - \varphi_{\text{Pt}}) \tag{3}$$

With the aid of equation (3) an arbitrary situation may be chosen as a reference point. In a number of cases it is possible to determine the composition of solution A at which the two phases (Pt and A) do not carry free charges. This situation is called the zero point of *charge*, or isoelectric point, and is often taken also as the zero point of *potential* difference between the two phases. Though this may be a useful assumption, it neglects the potential difference that may exist between two phases as a consequence of orientation of dipoles and polarization of atoms in the surface layers.

E. Galvani and Volta Potentials; χ-Potential

Lange and Koenig [(4); see also (5)] have given a clear analysis of the situation. Consider a condensed phase bounded by a vacuum. The electric potential just outside the phase, that is, out of the reach of short-range forces, is independent of the nature of the charge carrier which is used to measure it and consequently practically accessible and well defined. It is called the *Volta potential* (symbol ψ) and plays an important role in electron emission. A survey of methods for measuring Volta potentials was recently given by Möhring (6).

The potential inside the condensed phase has been termed *Galvani potential* (symbol φ) by Lange. It differs from the Volta-potential by an amount χ, the potential jump at the phase boundary:

$$\varphi = \psi + \chi \tag{4}$$

Both φ and χ depend upon the choice of the charge carrier. There are a few theoretical calculations of the potential jump, χ. They assume implicitly the existence of a nonmaterial charge carrier and are essentially calculations of the distribution of nuclei and electrons near the surface. How uncertain they are is shown clearly in the large variation in calculated χ-potentials of water: Verwey (7) found 0.5 volts (water negative) for the boundary water-vacuum. Tal'yanskiĭ (8) also concluded that the water molecules are preferentially oriented with their positive sides to the surface. Passoth (9), however, found 0.28 volts; Frumkin *et al.* (10), 0.1–0.2 volts; and Hush (11), 0.30 volts—all with water positive with respect to vacuum.

At the phase boundary of two condensed phases we distinguish the two Galvani potentials φ_{I} and φ_{II} and their difference $\chi_{\text{I, II}}$.

$$\chi_{\text{I, II}} = \varphi_{\text{I}} - \varphi_{\text{II}} \tag{5}$$

The interfacial potential jump $\chi_{\text{I, II}}$ is not equal to $\chi_1 - \chi_2$ because the molecules of both phases are rearranged at the interface as a consequence of the contact between the phases (Fig. 1). Frequently $\chi_{\text{I, II}}$ is divided into two contributions: one to be called $D_{\text{I, II}}$ and based upon the distribution of free charges, and the other to be called χ (the χ-potential in the restricted sense) and connected with polarizations and orientations of neutral molecules. At the zero point of charge $D_{\text{I, II}} = 0$, but χ usually has a finite value.

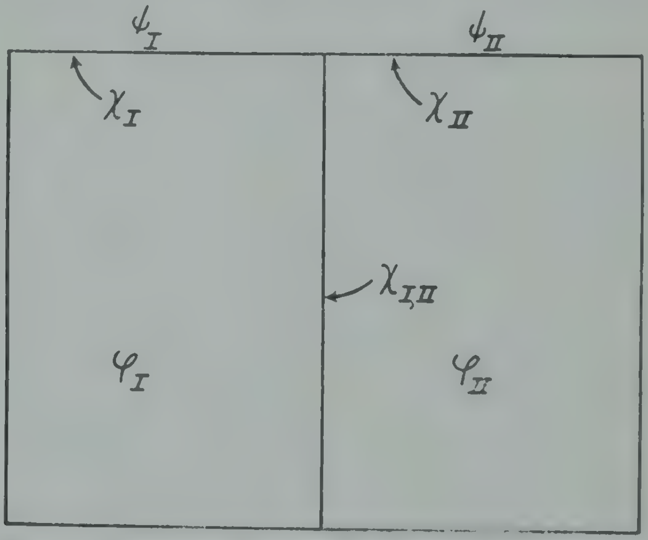


FIG. 1. Diagram illustrating Galvani (φ), Volta (ψ), and χ-potentials

F. Potential-determining Ions

If ions of type *i* occur in both phases and equilibrium exists, the electrochemical potential η_i of these ions has the same value throughout the whole system:

$$\eta_i^{\mathrm{I}} = \eta_i^{\mathrm{II}} \tag{6}$$

or, with equation (1):

$$\mu_i^{\mathrm{I}} + z_i e \varphi^{\mathrm{I}} = \mu_i^{\mathrm{II}} + z_i e \varphi^{\mathrm{II}} \tag{7}$$

Assuming phase II to be a solid with fixed composition (pure metal, oxide, sulfide, halide, etc.) and phase I to be a solution, dilute with respect to the ions (i), μ_i^{II} is a constant, and

$$\mu_i^{\mathrm{I}} = (\mu_i^{\mathrm{I}})_0 + kT \ln c_i \tag{8}$$

where $(\mu_i^{\mathrm{I}})_0$ is the standard potential of i ions, c_i their concentration in the bulk of phase I, and k the Boltzmann constant.

Substituting this in equation (7) we obtain the Nernst equation (9), which is seen to be valid not only for the potential difference between a metal and a solution, but also for oxides or salts in equilibrium with solution.

$$\varphi^{\mathrm{I}} - \varphi^{\mathrm{II}} = \text{constant} + \frac{kT}{z_i e} \ln c_i = \text{constant} + \frac{RT}{z_i F} \ln c_i \tag{9}$$

The ions of type i, for which equation (9) is valid, have been called *potential-determining ions* by Lange and Koenig (4). The Ba^{++} and SO_4^{--} ions in the system $BaSO_4$-solution, cited in Section II above, are examples of type i.

The χ-potential (in the restricted sense) is only based upon short-range forces. It may therefore be assumed to be independent of the distribution of free charges near the interface. With this assumption, the double-layer potential $D_{\mathrm{I,\,II}}$ is found also to obey a Nernst equation:

$$D_{\mathrm{I,\,II}} = \varphi^{\mathrm{I}} - \varphi^{\mathrm{II}} - \chi = \text{constant} + \frac{RT}{z_i F} \ln c_i \tag{10}$$

If the concentration of i ions is not very small, deviations from the simple logarithmic equation appear. It is customary to correct for these deviations by introducing an activity coefficient in the logarithmic term, but it should be realized that part of the deviations may just as well be attributed to the liquid junction.

G. Oil-Water and Air-Water Interfaces

Let us consider the distribution of an electrolyte (monovalent for simplicity) between two immiscible liquid phases (further denoted as oil and water) in equilibrium. Equality of the electrochemical potentials of the ions in both phases leads to

$$\eta_+^{\mathrm{oil}} = \eta_+^{w}$$

or:

$$(\mu_+^{\text{oil}})_0 + kT \ln c_+^{\text{oil}} + e\varphi^{\text{oil}} = (\mu_+^{w})_0 + kT \ln c_+^{w} + e\varphi^{w} \tag{11}$$

and

$$\eta_-^{\text{oil}} = \eta_-^{w}$$

or:

$$(\mu_-^{\text{oil}})_0 + kT \ln c_-^{\text{oil}} - e\varphi^{\text{oil}} = (\mu_-^{w})_0 + kT \ln c_-^{w} - e\varphi^{w} \tag{11a}$$

By subtracting the two equations (11) and (11a) from each other and taking into account that $c_+ = c_-$, one finds:

$$\varphi^{\text{oil}} - \varphi^{w} = \frac{(\mu_+^{w})_0 - (\mu_+^{\text{oil}})_0 + (\mu_-^{\text{oil}})_0 - (\mu_-^{w})_0}{2e} \tag{12}$$

The Galvani potential difference is independent of the concentration of electrolyte.

When the nonaqueous phase is a nonpolar hydrocarbon oil or air, the electrolyte concentration in it is zero or is so small as not to be sufficiently well defined to influence the potential difference. The distribution of charge and potential in the aqueous side of the boundary is in that case governed by the difference in adsorption of the two kinds of ions at the interface.

Incidentally the air-water interface is a good example of the determination of the Volta potential [see e.g. reference (12)]. If one electrode is placed in the air close to the surface and a second one connected by means of a saturated KCl liquid junction to the aqueous solution, the potential difference between these two electrodes changes parallel to the Volta potential of the solution. Adsorption of surface-active electrolytes and nonelectrolytes and spreading of amphipolar substances changes the Volta potential markedly. This change in Volta potential is equal to a change in the total χ-potential as the Galvani potential may be assumed to be unaffected by the small concentrations involved. The χ-potential at the water-air interface usually contains contributions from oriented dipoles and also from free ions.

IV. Ionic Double Layer

As stated in Section II, electrophoresis occurs only in the presence of freely mobile charges. It is highly sensitive to the amount and spatial distribution of these charges. Oriented dipoles and polarized molecules, giving rise to the χ-potential jump, do not lead to electrophoresis and for our problems are important only insofar as they influence the distribution of free charges in the ionic layers.

Our next aim will therefore be a closer study of the ionic double-layer potential $D_{\text{I, II}}$. Not to complicate matters unnecessarily, we shall provisionally consider only double layers at flat (noncurved) interfaces.

A. The Diffuse Double Layer

The ionic double layer consists of two layers of charges of opposite sign. In most cases one of the layers is localized in a plane at the transition between the phases, whereas the other one has the character of a space charge, extending fairly far into one of the two phases.

In the mathematical treatment the first-named layer is usually represented as smeared out in a mathematical plane. This is not as crude an approximation as it might seem to be, for often the surface charge is carried by electrons or by potential-determining ions. They are not localized, so that one might say that the excess charge is shared more or less equally between all electrons or potential-determining ions near the interface.

In the beginning of the development of the double-layer theory, the countercharge was represented as a space charge (extension not specified) in von Helmholtz' treatment of electrokinetics (13), as a surface charge by Perrin (14), and again as a space charge, carried by dimensionless ions, by Gouy (15) and by Chapman (16). Their treatment still furnishes the basis of modern double-layer studies. Stern (17), Grahame (18), and others have refined the Gouy-Chapman treatment by introducing finite size, hydration, etc., of the ions. We shall first follow the Gouy-Chapman treatment. A schematic picture of their double-layer model is given in Fig. 2, showing at the same time the potential distribution. The χ-potential jump, which ought to be superimposed on the double-layer potential as a sharp jump near the interface, has been omitted; the surface charge is assumed to be negative. Positive ions are attracted to the surface, negative ones are pushed away. The average number of ions (i) per unit volume at a distance

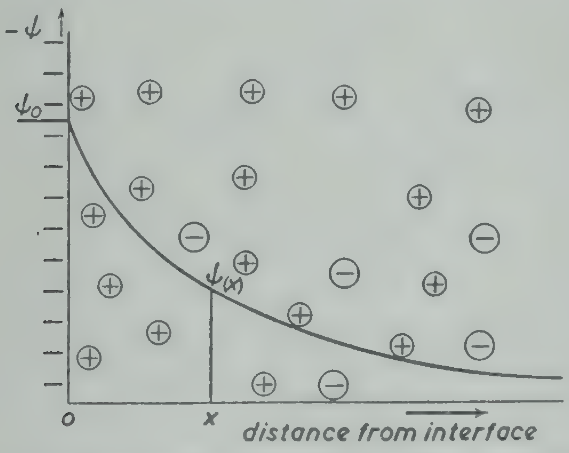


Fig. 2. Distribution of ions and potential in the diffuse double layer, omitting the χ-potential jump.

x from the interface $n_i(x)$ is connected with the average potential $\psi(x$ by a Boltzmann equation:

$$n_i(x) = n_i(\infty)\exp\left(-\frac{z_i e\psi(x)}{kT}\right) \tag{13}$$

where $n_i(\infty)$ is the number of ions in the bulk of the solution.

In the treatment of properties of strong electrolytes where equation (13 is also used, it has been criticized by Fowler (19) and others; see also: Onsager (20), Kramers (21), Fowler and Guggenheim (22), and especially Kirkwood (23). The objection is that in the exponential $z_i e\psi(x)$ ought to be the work done in bringing an ion to the distance x. However, if it is known that the ion is at x, it will influence the distribution of the ions in its neighborhood, so that the potential at x will deviate from the mean potential $\psi(x)$. Casimir (24) has pointed out that application of equation (13) is justified when large particles or charged surfaces with very many elementary charges are considered and when the electrolyte concentration is not too high. The objections are quite valid in the theory of electrolytes, where double layers (ionic atmospheres) around separate ions are considered.

The potential distribution in the double layer is connected with the space charge, $\rho(x)$, by Poisson's equation (14). This equation is the mathematical equivalent of the physical picture that $4\pi/\epsilon$ lines of force emanate from an electrostatic unit of charge in a medium with dielectric constant ϵ.

$$\operatorname{div}\,[\epsilon \operatorname{grad} \psi(x)] = -4\pi\rho(x) \tag{14}$$

If we assume ϵ to be a constant (see, however, below) and remember that in our model $\psi(x)$ changes only in the x-direction, equation (14) takes the simple form:

$$\frac{d^2\psi(x)}{dx^2} = -\frac{4\pi\rho(x)}{\epsilon} \tag{15}$$

The charge density is built up from the ionic charges or:

$$\rho(x) = \sum_i z_i e n_i(x) \tag{16}$$

The relations (13), (15), and (16) can be combined into the so-called Poisson-Boltzmann equation (17), which forms the basis of the double-layer theory:

$$\frac{d^2\psi(x)}{dx^2} = -\frac{4\pi}{\epsilon}\sum_i z_i e n_i(\infty)\exp\left(-\frac{z_i e\psi(x)}{kT}\right) \tag{17}$$

In the Debye-Hückel theory of strong electrolytes the analogous equation is solved by expanding the exponentials, retaining only the first two terms. This is a reasonable approximation provided $z_i e\psi(x)/kT < 1$ or $\psi(x) < 25/z_i$ millivolts at room temperature. Applying this approximation, and taking into account the electroneutrality far from the interface

$$\sum z_i e n_i(\infty) = 0 \tag{18}$$

equation (17) is transformed into:

$$\frac{d^2\psi(x)}{dx^2} = \kappa^2\psi(x) \tag{19}$$

in which

$$\kappa^2 = \frac{4\pi e^2 \sum\limits_i n_i(\infty) z_i^2}{\epsilon kT} \tag{20}$$

The solution of equation (19)

$$\psi(x) = \psi(0) \exp(-\kappa x) \tag{21}$$

shows that $1/\kappa$ has the dimension of a length and denotes that value of x for which $\psi(x) = \psi(0)/e$. Therefore $1/\kappa$ is a good measure for the extension of the double layer and is often called "thickness of the double layer."

In colloidal systems the application of the Debye-Hückel approximation (19) is frequently not justified. In the first place the underlying assumption $z_i e\psi(x)/kT < 1$ is mostly not fulfilled in the major part of the double layer. Potentials in the double layer may be as high as several hundred millivolts. Secondly, experiments on electrophoresis and on the stability of colloids reveal a pronounced difference between the influence of positive and negative ions. In equations (19) and (20), however, they appear in a completely symmetrical way, whereas the complete exponentials of equation (17) are indeed quite asymmetric with respect to positive and negative values of $z_i\psi_0$. It is therefore necessary to make use of the complete equation (17) for colloidal systems.

A first integration may be carried out after multiplying both sides of equation (17) by $\frac{2d\psi(x)}{dx}$. Making use of the boundary conditions

$$\psi(\infty) = 0 \quad \text{and} \quad \left(\frac{d\psi(x)}{dx}\right)_{x=\infty} = 0$$

integration leads to

$$\left(\frac{d\psi(x)}{dx}\right)^2 = \frac{8\pi kT}{\epsilon} \sum_i n_i(\infty)\left(e^{-\frac{z_i e\psi(x)}{kT}} - 1\right) \tag{22}$$

or for a solution of a $z_+ - z_-$ valent electrolyte

$$\frac{d\psi(x)}{dx} = \mp\sqrt{\frac{8\pi nkT}{\epsilon z_+ z_-}}\sqrt{z_- e^{-\frac{z_+ e\psi(x)}{kT}} + z_+ e^{\frac{z_- e\psi(x)}{kT}} - z_+ - z_-} \tag{23}$$

where $n = z_+n_+(\infty) = z_-n_-(\infty)$ is the concentration expressed in elementary charges per unit volume. The plus sign is valid for a negativel charged surface, the minus sign for a positive surface.

For a symmetrical $z - z$ valent electrolyte equation (23) can be written

$$\frac{d\psi(x)}{dx} = -\sqrt{\frac{8\pi nkT}{\epsilon z}}\left\{e^{\frac{ze\psi(x)}{2kT}} - e^{-\frac{ze\psi(x)}{2kT}}\right\} \qquad \text{(24}$$

The condition of electroneutrality in the whole system requires that an plane parallel to the interface cut the double layer into two layers carryin equal but opposite charges. Calling the sum of the surface charge σ at the interface and the space charge between $x = 0$ and x, $\sigma(x)$, this conditio leads to

$$\sigma(x) = -\int_x^\infty \rho(x)dx \qquad \text{(25}$$

Using the Poisson equation (15) to substitute for $\rho(x)$ and carrying out the integration we find:

$$\sigma(x) = +\int_x^\infty \frac{\epsilon}{4\pi}\frac{d^2\psi(x)}{dx^2}\,dx = -\frac{\epsilon}{4\pi}\frac{d\psi(x)}{dx}$$
$$= \pm\sqrt{\frac{\epsilon nkT}{2\pi z_+z_-}}\sqrt{z_-e^{-\frac{z_+e\psi(x)}{kT}} + z_+e^{+\frac{z_-e\psi(x)}{kT}} - z_+ - z_-} \qquad \text{(26)}$$

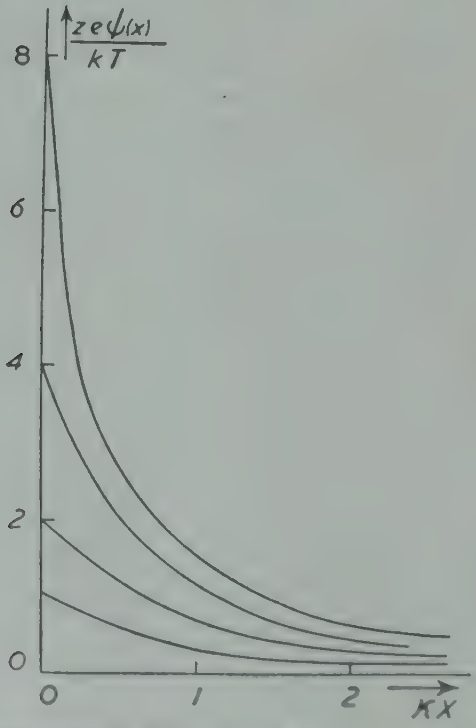


FIG. 3. Potential in the diffuse double layer for various surface potentials and symmetrical z-z valent electrolytes as a function of the distance from the interface.

an equation which for $x = 0$ relates the surface charge $\sigma = \sigma(x = 0)$ with the surface potential $\psi_0 = \psi(x = 0)$.

Although a second integration of equation (23) can be carried through, at least when z_+ and z_- are equal or not larger than 2, the resulting equations are rather complicated and will not be needed here. Figure 3 gives a graphical representation of the potential as a function of the distance from the interface. In this figure the dimensionless units $ze\psi/kT$ and κx are used, which means, that the potentials are expressed in units of $25/z$ millivolts and the distances in units $1/\kappa$. The values of $1/\kappa$ can be calculated from equation (20). For water at 25°C. and symmetrical $z - z$ valent electrolytes, this equation can be written: $1/\kappa = 96/z\sqrt{c}$ in which $1/\kappa$ is expressed in Ångström units and c in millimoles per liter. For monovalent electrolytes, $1/\kappa$ is about 10 Å. for 0.1 molar, 100 Å. for millimolar, and 1000 Å. for 10^{-5} molar solutions.

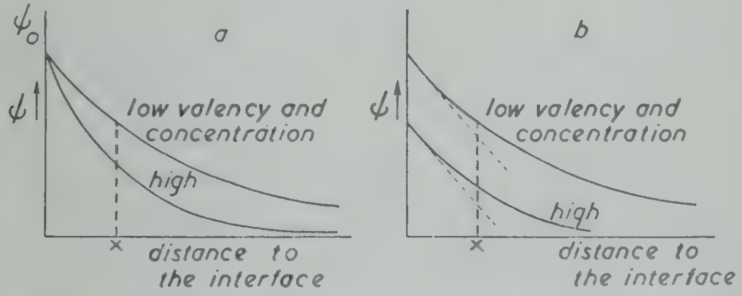


FIG. 4. The part of the double layer that is responsible for electrophoresis becomes less important for higher concentration and valence of electrolyte. a. Constant surface potential. b. Constant surface charge.

The fact that the double layer is thinner and the potential drop steeper in more concentrated solutions and in solutions containing ions of higher valency than in dilute ones is of great importance for electrophoresis. There are many indications that in electrophoresis the first few layers of molecules near the interface do not move and that electrophoresis is determined by the outer part of the double layer. Figure 4 shows schematically how, for constant surface potential and (still more pronounced) for constant surface charge, the potential at a distance x from the interface is lower, the thinner the double layer. Consequently electrophoresis will be slower, the higher the ionic strength.

Experimental checks of the double layer theory are usually based upon the relation between surface charge and potential (equation 26). Extensive data on the interface mercury-aqueous solution are available through electrocapillary work (18) and on the interface AgI-aqueous solution by determination of the adsorption of potential-determining ions (I^-, Ag^+)

combined with potential measurements on a cell with a silver-silver iodide electrode (25, 26). Double layers on many other materials have been investigated.

Equation (26) turns out to be valid only for low concentrations of electrolyte, low potentials, and low charges. For higher charges equation (26) predicts an exponential relation between charge and potential, whereas a more nearly linear relation is found. This discrepancy may be ascribed to the finite size of the ions as was first clearly recognized by Stern (17). Even at a concentration in the bulk of the solution as low as $\frac{1}{100}$ molar and a surface potential of 250 millivolts (mv), which is not extremely high, the concentration of counterions near the surface is, according to the Boltzmann equation,

$$\text{Conc. at surface} = c_\infty \exp\left(\frac{e\psi}{kT}\right) = \frac{1}{100}\exp\frac{250\text{ mv}}{25\text{ mv}} = 220\text{ molar}$$

—clearly an impossible value. For the innermost parts of the double layer, the Boltzmann equation can obviously not be accepted without considerable corrections.

B. Influence of the Finite Size and Specific Properties of Ions and Other Corrections of the Gouy-Chapman Theory

In the preceding paragraph it has been shown that the finite size of ions necessitates corrections of the diffuse double-layer theory. These corrections are much more important near the interface, where concentrations may be high, than in the bulk of the solution. Stern (17) was the first to introduce a correction of this kind by treating the adsorption of the first layer of ions separately and using the Gouy-Chapman theory for the remaining part of the double layer. In the first layer, electrical effects, size of the ions, and specific adsorption could be taken into account, thus forming a basis for the explanation of specific differences between ions of the same valence in their influence on electrophoresis, double-layer capacity, etc. A fine elaboration of Stern's ideas was given by Grahame (18).

The Russian school (27–30) paid attention to the influence of the discreteness of the charges in the Stern layer on the amount of specifically adsorbed ions and the accompanying potential distribution. Recently their results were confirmed and extended by Grahame (31, 32).

Bikerman (33) applied a number of corrections to the Boltzmann equation, one of them for ionic volume, thus automatically cutting out the absurdly high concentrations that may occur in the Gouy-Chapman formalism. In addition he considered specific adsorption for the first layer of ions.

Parsons (34), while using the Stern approach, showed that the specifically adsorbed ions follow an Amagat equation rather than a Langmuir one. Loeb (35) and Williams (36) took the Debye-Hückel interaction between the individual ions in the double layer into account.

Freise (37) treated the diffuse double-layer structure from the point of view of ideal mixing and found deviations from the Gouy-Chapman theory which could be interpreted as a consequence of the finite size of solvent molecules and ions.

Conway *et al.* (38), Hasted and co-workers (39), Grahame (40), and Booth (41) considered the dielectric constant in the double layer. From their investigations it appeared that the dielectric constant may be considerably lowered in the strong electric field in the double layer. This change in dielectric constant has, however, a relatively minor influence on the relation between surface charge (σ) and surface potential ψ_0. Bolt (42) concluded that most of the corrections in the diffuse part of the double layer are mutually compensating, so that for many practical purposes they may be neglected.

This, however, is not the case for the correction for finite size and specific adsorption of the first layer of ions. For these effects we shall apply the Stern treatment as modified by Grahame.

The surface charge is again assumed to be smeared out in a mathematical plane. The first layer of ions in the solution is found at a distance δ from this plane. Beyond δ the double layer follows the Gouy-Chapman pattern. In Stern's own treatment there is some uncertainty as to how the first layer of ions and the diffuse double layer connect with each other. Grahame distinguishes two planes in the double layer, parallel to the surface, which he denotes as the inner and the outer Helmholtz plane. The outer Helmholtz plane lies at the distance of closest approach to the surface for hydrated ions, still belonging to the diffuse double layer. In the absence of specific adsorption, no centers of ions occur between the outer Helmholtz plane and the surface charge. The inner Helmholtz plane is the plane in which specifically adsorbed and presumably dehydrated ions are found. The distribution of ions and the course of the potential according to Grahame's picture are given schematically in Fig. 5, a for negative surface charge without specific adsorption. Figure 5, b and c, shows the situation as modified in the presence of weak and strong specific adsorption of anions. In the last case, the specific adsorption is so strong, that not only the charge of the diffuse layer is positive but also that of the wall. Figure 5, d and e, shows cases with specific adsorption of cations. With strong adsorption of cations, the charge in the diffuse double layer may become reversed and electrophoresis would be directed to the negative pole.

Quantitative treatment of the case without specific adsorption is rather simple. The double layer may be represented by a system of two condensers

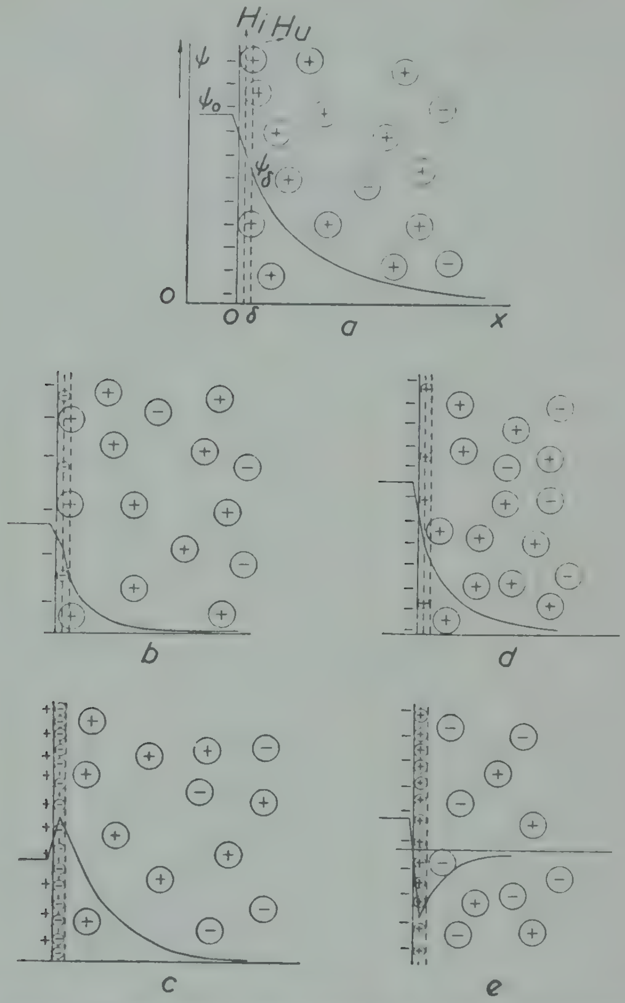


FIG. 5. Distribution of ions and potential in the double layer according to Grahame (18). a. No specific adsorption. b. Specific adsorption of anions. c. Strong specific adsorption of anions. d. Specific adsorption of cations. e. Strong specific adsorption of cations.

in series. The first one, the "molecular condenser," has the plane of the charge on the wall as one of its plates and the plane δ as the other one. Its capacity can be written as:

$$C_m = \frac{\epsilon'}{4\pi\delta}$$

where ϵ' is the effective dielectric constant in this layer. The diffuse double layer has a capacity

$$C_d = \frac{\sigma}{\psi(x = \delta)}$$

in which $\sigma = \sigma_d$ (no charges between $x = 0$ and $x = \delta$). The total capacity

C is found from

$$\frac{1}{C} = \frac{1}{C_m} + \frac{1}{C_d} \tag{27}$$

and is also equal to the ratio of surface charge (σ) to total potential drop (ψ_0).

The capacity of the diffuse part of the double layer can be calculated as a function of the double layer charge σ by means of equation (26). For low charge it is equal to $\epsilon/4\pi \frac{1}{\kappa}$, but it increases strongly with increasing potential.

Stern considered the capacity of the molecular condenser as a constant, but Grahame has shown that it can be more satisfactorily treated as a function of the surface charge, i.e. as a function of the field strength near the surface. In order to determine C_m, Grahame (18, 43) applied equation (27) to the double layer on mercury in aqueous NaF solutions. When the electrolyte concentration is high, the diffuse part of the double layer is thin ($1/\kappa$ small) and possesses a high capacity. Consequently, according to equation (27) the total capacity is mainly determined by C_m. For this reason Grahame used the total capacity of the double layer in 1 *M* NaF to calculate C_m. Figure 6 shows the result obtained. Conversely with the aid of C_m thus found, the total double-layer capacity in lower concentrations of NaF can be calculated, supposing C_m independent of the electrolyte concentration.

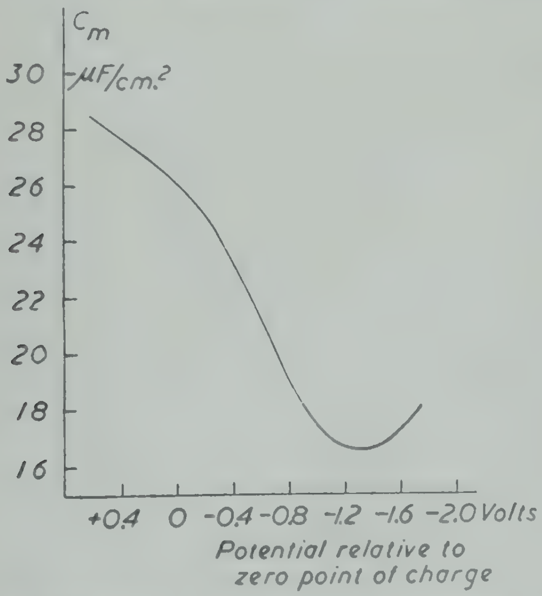


FIG. 6. **Capacity** of the molecular condenser on mercury in **1.0** *M* NaF solutions.

In this way excellent agreement with experiments is obtained. Figure 7 shows the calculated and experimental results for 0.001 *M* NaF.

Grahame (44) determined data for many other electrolytes. Similar results have been obtained for the double layers on mercury in water by Ross McDonald (45), G. and E. Darmois (46), Vorsina and Frumkin (47), Watanabe *et al.* (48); on AgI in water, by Overbeek and Mackor (49, 50), and Lijklema (26); on Ag_2S in water, by Freyberger and de Bruyn (51) and Iwasaki and de Bruyn (52).

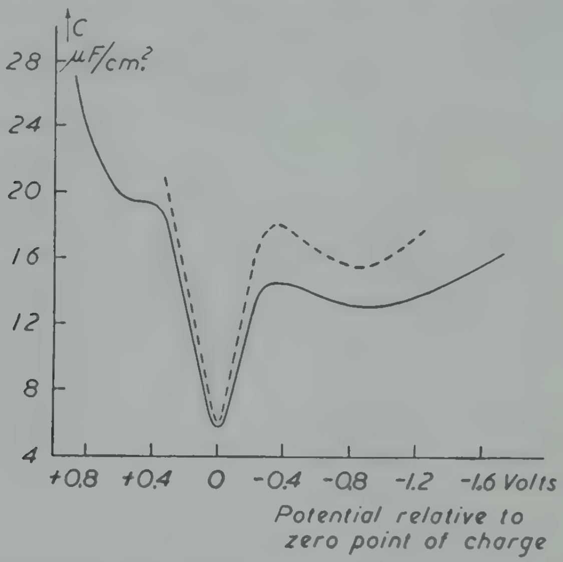


FIG. 7. Total capacity of the double layer on mercury in 0.001 *M* NaF. Dashed line calculated with equation (27). Solid line based on experiments (18, 43).

The picture so far developed already includes some specific effects. The capacity of the molecular condenser (Stern layer) is expected to be dependent on size and polarizability of the ions, and thus to follow the lyotropic order.

According to Grahame's data on mercury the capacity of the molecular condenser increases systematically with atomic number and valence (that is in the lyotropic order) (53):

$$Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$$

$$Ca^{++} < Sr^{++} \simeq Ba^{++}, \text{ etc.}$$

The differences in capacity between different ions are so small that they are distinguishable only with very accurate experiments.

Lijklema's work on AgI shows the following order for the cations

$$Li^+ < K^+ < Rb^+ < NH_4^+ < Co^{++}$$

$$\simeq Cd^{++} < Mg^{++} \simeq Ba^{++} < La^{+++} < Th^{++++}$$

and for the anions

$$F^- < ClO_4^- < NO_3^- < SO_4^{--}$$

The effects on AgI, though still small, as shown for instance in Fig. 8, are somewhat greater than those on mercury.

Although specific adsorption of ions is of rather common occurrence, there are only very few cases where a quantitative interpretation has been made. A good example is the adsorption of Cl⁻ on mercury as investigated by Grahame (18). If the mercury is sufficiently negative no adsorption of Cl⁻ takes place (because it is repelled too strongly by the electric field) But even with a weak negative charge on the mercury, Cl⁻ is already adsorbed specifically; on positively charged mercury the Cl⁻ adsorption is so strong that the charge of the diffuse double layer is also positive (see Fig. 5c). This strong specific adsorption of Cl⁻ (and other halides) on mercury is obviously related to the strong interaction between mercury and halides in mercury halides.

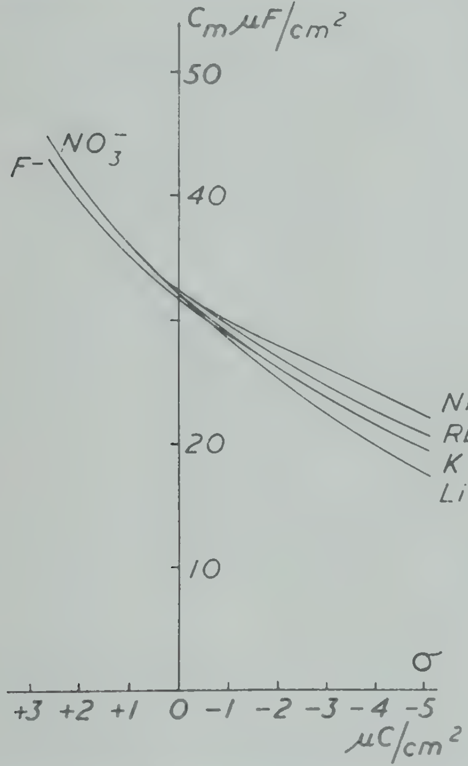


FIG. 8. Capacity of molecular condenser on silver iodide according to Lijklema (26).

Very obvious examples of specific adsorption are found in those cases where adsorbed ions invert the sign of the charge in the diffuse double layer. Many complex ions (Co-, Pt-complexes) and paraffin-chain ions have this property. In this connection it should be borne in mind that such a charge reversal can never be brought about by purely electrostatic forces.

Paraffin-chain ions are specifically adsorbed at all interfaces between water and other phases because the paraffin tail is "squeezed out" as a consequence of the strong attractions among the water dipoles. This effect is particularly evident at water-"oil" interfaces, where by "oil" we mean any nonpolar or slightly polar organic liquid.

In the simplest cases the potential difference between water and oil is determined by the distribution of an electrolyte between the two phases (as treated in Section III, G, equations (11 and 12)). In such circumstances there will be a diffuse double layer in each of the phases and no surface charge at all at the phase boundary [see Verwey and Niessen (54)]. Quite often the concentration of ions in the oil phase will be very low, and the amount of charge in the double layer very small as well. Even a relatively weak specific adsorption of ions at the interface will change the picture to the familiar one of a surface charge at the phase boundary and a space charge on its aqueous side.

An interpretation of the capacity of the molecular condenser in terms of dielectric constant and distance between the layers of charges is as yet hardly possible. A normal value for this capacity is 20 μF./cm.^{2}. With $\epsilon' = \epsilon = 80$ this would correspond to a distance δ of 35 Å. and with $\epsilon' = 2$ to $\delta = 1$ Å. Both values are quite improbable, and the truth lies somewhere in between—probably at a dielectric constant of 4 to 10 and a distance of 2–5 Å.

The lyotropic order for monovalent ions corresponds to a stronger hydration for the smaller ions. Strong hydration implies at the same time a high effective ionic radius (high value of δ) and a small dielectric constant, both factors working in the direction of a small capacity. The large capacity for polyvalent ions is more difficult to interpret. Among other effects one should take into consideration that the lines of force spreading from a polyvalent ion hit the interface on a larger area than those of a monovalent ion. The lines of force will therefore pass regions relatively far from any ion, where the water molecules are relatively free and the dielectric constant is high.

This discussion shows that interpretation of the capacity of the molecular condenser is connected with orientation and fixation of molecules very near the interface. Mackor (25) has pointed out that an equally valid interpretation is based on variability of the χ-potential. Indeed the seat of at least a part of the χ-potential is in the same layers of molecules that form the dielectric for the molecular condenser. It is more a question of convenience than one of principle how the contribution of these molecules to the electric field is split up between their influence on the χ-potential and on the dielectric constant of the molecular condenser. Mackor's way of interpretation has the advantage that the dissymmetry in the capacity at the two sides of the zero point of charge, which is nearly always found, can be related to a preferential orientation of the water molecules at the uncharged surface. More obvious cases of a shift of the χ-potential are found in the influence of electroneutral materials (acetone, urea, thiourea, resorcinol) on double-layer properties. In preferential adsorption at the interface such materials are as a rule oriented, thus causing an extra potential difference between the two phases and a corresponding shift in the zero point of charge. For example the zero point of charge for AgI in water moves in the direction of lower I^- concentrations by addition of acetone (55).

C. The Double Layer at Curved Interfaces

In actual electrophoresis of finite particles the interface between particle and medium cannot be completely flat. But if the radius of curvature of the interface is much larger than the thickness of the double layer ($1/\kappa$), the treatment of the double layer at a flat interface (as given above in Section IV, A and B) forms a very good approximation. However, for small particles with a size of the same order as the thickness of the double layer or even smaller, an explicit treatment of the double layer at curved interfaces is desirable. There is a good deal of literature on spherical double layers and a few articles on cylinders. It is typical for these cases that there is relatively more volume available far away from the interface than close to it. Consequently, those parts of the double layer where the potential is low are relatively more important than the flat double layer and the Debye-Hückel approximation is more successful for curved double layers than for flat ones. Going on to particles with a very small radius, this same effect explains the success of the Debye-Hückel theory for electrolytes, although potentials at the ionic surfaces are frequently higher than 100 millivolts.

1. Spherical Interfaces

The Poisson-Boltzmann equation (17)

$$\operatorname{div}\operatorname{grad}\psi(r) = -\frac{4\pi e}{\epsilon}\sum_i n_i(\infty)z_i\exp\left(-\frac{z_ie\psi(r)}{kT}\right) \tag{17'}$$

takes the following form for spherical symmetry

$$\frac{1}{r^2}\frac{d}{dr}\left(r^2\frac{d\psi(r)}{dr}\right) = -\frac{4\pi e}{\epsilon}\sum_i n_i(\infty)z_i\exp\left(\frac{-z_ie\psi(r)}{kT}\right) \tag{28}$$

For low potentials Debye and Hückel (56) have given the following approximated solution of equation (28):

$$\psi(r) = \psi(a)\,\frac{a}{r}\,e^{\kappa(a-r)} \tag{29}$$

in which a denotes the radius of the sphere. The surface charge density σ is then given by

$$\sigma = -\frac{\epsilon}{4\pi}\left(\frac{d\psi(r)}{dr}\right)_{r=a} = -\frac{\epsilon\psi(a)}{4\pi a}\,(1 + \kappa a) \tag{30}$$

and the total charge on the spherical particle by:

$$Q = 4\pi a^2\sigma = -\epsilon a\psi(a)(1 + \kappa a) \tag{31}$$

Just as in the case of flat double layers it is insufficient for most purposes to use only the first two terms of the power series and it is desirable to find more precise solutions of the Poisson-Boltzmann equation (28). However, an integration of the total spherical Poisson-Boltzmann equation in closed form is not possible. Various useful elaborations are given in the literature. In 1928 Müller (57) elaborated a procedure for numerical integration of equation (28), or rather of the special form it takes for symmetrical electrolytes. The number of solutions that have been worked out, however, was insufficient to make the method of much practical value.

Gronwall *et al.* (58) and La Mer *et al.* (59) gave a solution of equation (28) in the form of a series expansion in terms of the dimensionless quantity $z^2e^2/\epsilon akT$ for $z - z$ valent electrolytes. Unfortunately only the first few terms of the series have been calculated, the series converging only for low values of the parameter; solutions based on these terms therefore are acceptable only in cases of still rather low surface charge and potential.

Mikulin (60) has shown that replacement of κ by (κ + constant$\cdot\psi$) in equation (29) leads to an approximate solution of the complete Poisson-Boltzmann equation of rather wide acceptability. The method, however, is rather laborious and still not quite exact.

The most practical way to solve equation (28) is found in the use of electronic computors. This amounts to mechanizing the tedious procedure of numerical integration so that solutions for a sufficient number of conditions can be evaluated. Hoskin (61) introduced this method. His results were for the greater part confirmed and extended by Loeb and associates (62). The computor calculations by Wall and Berkowitz (63) on polyelectrolytes (see Section IV, D) also contain data on spherical double layers for high potentials. Hoskin's data cover the values $ze\psi_0/kT = 1, 2, 4, 6, 8$ and $\kappa a = 1, 3, 5$, and 15. Loeb *et al.* extend these data to higher potentials and smaller values of κa and give accurate approximations for combinations of parameters not covered by the actual computations.

TABLE I

VALUES[a] OF $\frac{4\pi e}{\epsilon kT} \frac{z}{\kappa} \cdot \sigma$ FOR VARIOUS VALUES OF κa AND $\frac{ze\psi_0}{kT}$

$\frac{ze\psi_0}{kT}$	$\kappa a = 20$	$\kappa a = 10$	$\kappa a = 5$	$\kappa a = 2$	$\kappa a = 1$	$\kappa a = 0.5$	$\kappa a = 0.2$	$\kappa a = 0.1$
1	1.0913	1.1403	1.2386	1.5344	2.0297	3.0238	6.0155	11.010
2	2.4430	2.5359	2.7230	3.2910	4.2522	6.2019	12.132	22.087
3	4.3861	4.5144	4.7740	5.5698	6.9372	9.7562	18.495	33.322
4	7.4067	7.5614	7.8744	8.8428	10.530	14.080	25.376	44.896
5	12.271	12.444	12.791	13.878	15.785	19.878	33.310	57.170
6	20.218	20.403	20.772	21.929	23.965	28.377	43.327	70.864
7	33.273	33.465	33.848	35.041	37.138	41.678	57.395	87.544
8	54.773	54.967	55.359	56.569	58.677	63.214	79.008	110.34
9	90.202	90.397	90.794	92.013	94.106	98.580	113.95	144.95
10	148.60	148.80	149.20	150.42	152.50	156.87	171.58	201.08
11	244.89	245.09	245.48	246.70	248.77	253.03	267.09	294.58
12	403.63	403.83	404.23	405.44	407.49	411.68	425.18	450.71
13	665.34	665.53	665.94	667.14	669.17	673.33	686.38	710.29
14	1096.8	1097.0	1097.4	1098.6	1100.6	1104.8	1117.5	1140.2
15	1808.2	1808.4	1808.8	1810.0	1812.0	1816.1	1828.6	1850.5
16	2981.2	2981.4	2981.8	2983.0	2985.0	2989.0	3001.3	3022.5

[a] Computed by Loeb *et al.* (62).

By way of illustration we include Table I, taken from the work of Loeb *et al.* (62), giving the surface charge density σ as a function of $ze\psi_0/kT$ and κa.

A practical application of Hoskin's data can be found in the work of Stigter and associates (64, 65) on surface potential, charge, and other properties of soap micelles. Surface potentials appear to vary between 100 and 200 millivolts. Application of the Debye-Hückel expression would erroneously lead to potentials more than twice as high in several cases.

Combinations of diffuse double-layer theory around spherical particles with Stern corrections are as yet rarely used, but it is felt that in any case of practical interest, the quasi-flat Stern (or Grahame) molecular condenser may be combined with the spherical diffuse layer, because the thickness of the Stern layer is so small ($\delta \ll a$).

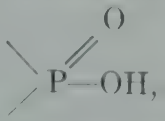
2. *Cylindrical Symmetry*

The distribution of ions in a diffuse double layer around a cylindrical particle has been treated for the case of small potentials by Dube (66) and by Gorin (67). Charge and potential are related through Bessel functions.

Fuoss *et al.* (68) have treated the case of infinitely thin rods surrounded by diffuse double layers consisting only of ions with a charge opposite to that of the rods themselves. The case is important for the behavior of polyelectrolytes.

D. Double Layers around Polyelectrolytes

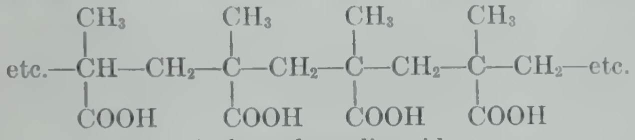
In the preceding sections we mainly considered systems in which the surface charge is generated by *adsorption*. There are other very important colloids in which the charge is formed by *dissociation*. These are nowadays called polyelectrolytes, but they are more or less identical with what used to be called hydrophilic colloids. Polyelectrolytes are molecules of colloidal dimensions containing a large number of ionizable groups like $-\mathrm{COOH}$, $-\mathrm{NH_2}$, $>\mathrm{P}(=\mathrm{O})-\mathrm{OH}$, etc. Proteins are an example of this group.



One might be inclined to consider H^+ and OH^- as the potential-determining ions for these systems. The Nernst equation (9), however, is not followed exactly because the particles cannot take up an indefinite amount of H^+ ions and saturation plays a role. Or, more formally, the chemical potential of the H^+ ions on the particles is not a constant but depends on the number already adsorbed.

The relation between charge and pH can be found experimentally by titration. Titration curves are more complicated than those of simple electrolytes, mainly on account of the mutual interaction amongst the charged groups.

Consider a polyelectrolyte molecule with carboxyl groups. The dissociation of the first few groups will give the molecule a negative charge, which will hamper the dissociation of the following groups. The carboxyl groups become more weakly acidic, the higher the degree of ionization. As the interaction between different charged groups is partly screened off by the ionic atmospheres around each of them, the interaction depends on the ionic strength of the solution and consequently titration curves change with ionic strength. At a fixed pH dissociation is stronger, the higher the salt concentration. In addition the form of polyelectrolyte molecules is often far from simple and not constant. If the polyelectrolyte is a long-chain molecule such as polymethacrylic acid its form in solution will be a loose coil, the shape of which is determined by statistics (69). The extension of the coil increases with increasing charge on the molecule as a consequence of growing mutual repulsion between the charged groups. The ionic strength affects the degree of swelling of the coil. A nonlinear polyelectrolyte may be either more like a sponge or network, and also subject to swelling and shrinking, or it may be a more compact and rigid structure as in many native proteins.



$$\text{etc.}-\underset{\underset{\text{COOH}}{|}}{\overset{\overset{\text{CH}_3}{|}}{\text{CH}}}-\text{CH}_2-\underset{\underset{\text{COOH}}{|}}{\overset{\overset{\text{CH}_3}{|}}{\text{C}}}-\text{CH}_2-\underset{\underset{\text{COOH}}{|}}{\overset{\overset{\text{CH}_3}{|}}{\text{C}}}-\text{CH}_2-\underset{\underset{\text{COOH}}{|}}{\overset{\overset{\text{CH}_3}{|}}{\text{C}}}-\text{CH}_2-\text{etc.}$$

Polymethacrylic acid

Only in the simplest cases are the charge-carrying groups all identical. In most actual cases there are groups of different nature and even of different sign present on one molecule.

No wonder that a theoretical treatment of the relation between charge and pH of polyelectrolytes is a complicated affair and, although a good deal of work has been accomplished, the subject is far from closed.

For a review, papers by Katchalsky [see, for example, (70)] are recommended.

An extremely rapid development in the field began in 1948 when Katchalsky, Künzle, and Kuhn (71), Hermans and Overbeek (72), and Overbeek (73) presented their work in this field at a symposium in Liège (Belgium) and Fuoss and Cathers (74) published a paper on polyvinyl pyridinium compounds. In these early papers the three main aspects of polyelectrolyte theory are clearly recognized. They are: (1) the distribution of the small ions at given charge and configuration of the polyelectrolyte molecule; (2) the relation of charge and configuration with pH and ionic strength; (3) the interpretation of experimentally accessible magnitudes such as viscosity, sedimentation, light scattering, electrophoresis, etc.

1. *Charge and Potential in and around Polyelectrolyte Coils*

The distribution of ions around a polyelectrolyte molecule differs from that in the double layers (as described above) insofar that quite often small ions penetrate within the polyelectrolyte molecule and that it surely is not always permissible to consider the charge on the polyelectrolyte molecule as smeared out.

A treatment that may serve as a good basis and allows a number of refinements considers a coiled polyelectrolyte molecule as a rather open spherical structure with a fixed charge smeared out uniformly in a sphere. The distribution of small ions is thought to be governed by the Boltzmann equation, which is valid both inside and outside this sphere. Outside the coil the solutions of the Poisson-Boltzmann equation for spherical symmetry are valid; inside the coil this equation has to be modified as follows to take the fixed charge into account:

$$\text{div grad } \psi(r) = -\frac{4\pi e}{\epsilon}\sum_i n_i(\infty) z_i \exp\left(-\frac{z_i e\psi(r)}{kT}\right) - \frac{4\pi\rho_0}{\epsilon} \qquad (32)$$

in which ρ_0 is the charge density of the fixed charges. The dielectric constant ϵ is assumed to have the same value as in the free solution. The integration constants of the solutions inside and outside the coil have to be chosen in such a way as to avoid discontinuities and infinities, and so that the total charge of the small ions just compensates the fixed charge of one coil. Hermans and Overbeek (72, 75) solved these equations, using the Debye-Hückel approximation. Kimball *et al.* (76) assumed the charge of the coil to be completely compensated within the coil by a constant excess of counterions and a deficit of co-ions just as in a Donnan equilibrium, no double layer being present outside the coil, with a discontinuous transition at the coil boundary. Although this model seems rather crude, it allows the use of the unapproximated Boltzmann equation and it gives a better approximation for the electrical potential within the coil than does the treatment by Hermans and Overbeek. More refined solutions have been obtained by Osawa and co-workers (77), by Fujita (78), and especially by Wall and Berkowitz (63), who solved equation (32) and the corresponding equation (28) by using an electronic computer. Figure 9 gives a comparison of three different solutions for a typical case.

Lifson (79) found an approximated analytical solution which in most cases comes very close to that of Wall and Berkowitz and which may be very useful for further work.

Other distributions than the homogeneous one for the fixed charge a have been considered by several of the authors mentioned above. The effect of replacing a homogeneous distribution by a Gaussian one appeared to result in quite minor alterations.

Künzle (80), in order to be able to take the distribution of the charges along the chain better into account, assumed the ionic atmospheres around the individual charges to be additive. Lifson (79) drew attention to the important interaction effect between neighboring charges. As long as the charge of the coil is rather low the charges will "avoid each other" and the actual interaction will be reasonably well described with a smeared-out distribution. When the charge density, however, is high and neighboring sites on the chain are of necessity charged simultaneously, the nearest neighbor interactions become quite important and have to be added separately to the interaction with more distant charged sites.

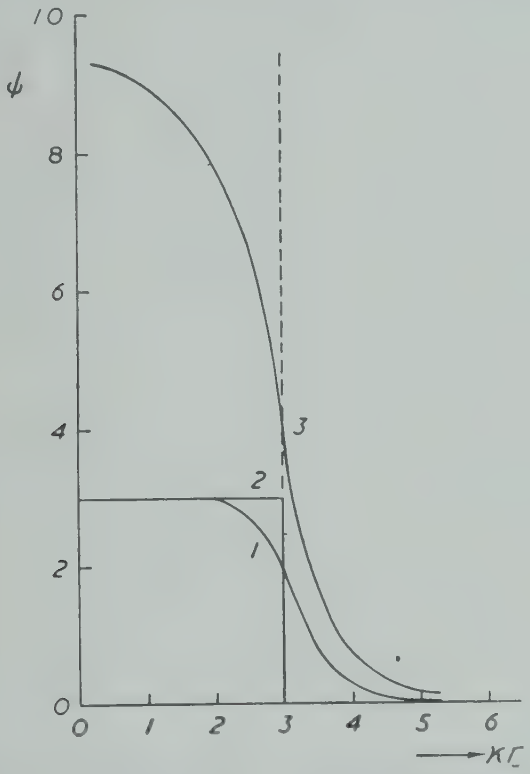


FIG. 9. Radial potential distribution in a polyelectrolyte coil according to: 1. Wall and Berkowitz (63) (exact solution). 2. Kimball *et al.* (76) (Donnan method). 3. Hermans and Overbeek (75) (Debye-Hückel approximation).

2. *Titration Curves of Polyelectrolytes*

In the absence of interaction between the different charged sites of a polyelectrolyte molecule, the titration curve can be predicted completely from the dissociation constants of the charged groups. In the simplest case, namely with a single type of charged groups, e.g. COO^-, the titration curve corresponds to that of a monovalent acid.

The interaction among the charged groups and the small ions can be expressed in terms of F_{el}, the electrical part of the free energy of the coil. This quantity can be calculated from the distribution of charge and potential in a straightforward way. In the Debye-Hückel approximation F_{el} is equal to

$$F_{el} = \tfrac{1}{2} \int \rho_0 \psi \, dV \tag{33}$$

where the integration has to include a sufficient volume to include all fixed charges.

The addition of one more elementary charge to the total fixed charge of the coil demands an amount of work, $\frac{dF_{el}}{dn}$, where n is the number of elementary charges of the coil. This amount of work has to be performed in addition to the normal chemical work of dissociation of the charged groups, and at this stage of dissociation the titration exponent pK is therefore modified as follows

$$K = K_0 \exp\left(-\frac{dF_{el}/dn}{kT}\right) \tag{34}$$

or

$$pK = pK_0 + \frac{dF_{el}/dn}{kT} \tag{34a}$$

Overbeek (73) treated this effect using the Debye-Hückel approximation and applying it to the titration curves of gum arabic. From the experimental titration curve F_{el} was determined and from F_{el} the size of the molecule. The radius of the coil was found to be about 100 Å. and to change with charge and ionic strength as predicted. Of course, every refinement used in the description of the double layer can be included in equations (34 and 34a). In the titration curves of proteins these same aspects occur, though in a more complicated way on account of the multitude of types of charged groups. These aspects are treated in the chapter by Linderstrøm-Lang and Nielsen in this volume.

V. Electrokinetic Potentials

So far we have treated charge and potential near the phase boundary but given little attention to their connection with electrophoresis. Charge and potential are equilibrium properties; electrophoresis and electrokinetics in general introduce the aspect of a tangential movement of the two phases along each other. The laws of viscous flow, which govern this tangential motion, are well known, but there exists great uncertainty about the actual value of the viscosity coefficient, η, in the double layer. The viscosity will be larger than in the free liquid for two reasons: (a) the high field strength; (b) short range interactions between the two phases at the phase boundary.

For solid or highly viscous liquid particles suspended in a liquid it is customary to take these effects roughly into account by assuming the existence of a "slipping plane," parallel to the actual phase boundary, but displaced somewhat to the side of the liquid. The liquid is assumed to be completely immobile between the phase boundary and the slipping plane, but to have its normal viscosity beyond this plane. In accordance with this view electrokinetic phenomena would be determined by the outer part of the electrical double layer and more particularly by the potential at the slipping plane, called the *electrokinetic potential* or *ζ-potential*.

The electrophoretic velocity, U, is given by the following equation:

$$U = A \frac{\epsilon\zeta}{\eta} E \qquad (35)$$

where E denotes the applied field strength; ϵ and η the bulk values of the dielectric constant and viscosity, respectively; and A is a constant, the value of which is given as $\frac{1}{4\pi}$ by von Helmholtz (13) and von Smoluchowski (81), $\frac{1}{6\pi}$ by Hückel (82), and still other values by Henry (83), Booth (84), Overbeek (85), and others.

Figure 10 shows that the ζ-potential is smaller than the potential ψ_0

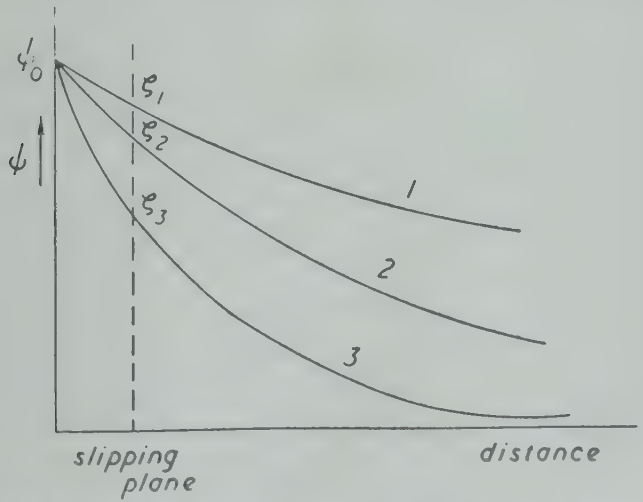


FIG. 10. Curves showing how the potential of the slipping plane decreases with increasing electrolyte content or valence (1 = low, 2 = intermediate, 3 = high ionic strength).

at the phase boundary and that ζ decreases strongly with increasing electrolyte content and valency, even when the surface potential remains constant. Qualitatively this picture is in good agreement with experimental facts. Streaming potentials and electroosmosis in glass capillaries [Freundlich and Rona (86), Rutgers and de Smet (87)] reveal that addition of electrolytes, which affects ψ_0 (or the total potential difference between the two phases) very little, nevertheless causes the ζ-potential to decrease markedly. Still it has not been possible to base a quantitative interpretation of electrokinetics upon this picture. Eversole and Boardman (88), for instance, calculated the distance between the phase boundary and the slipping plane from the electrolyte concentration–ζ-potential relations as found by different authors. In some cases the results can be explained by a constant thickness of the immobile layer, but the thickness varies between 8 and 100 Å. from one series of experiments to another.

Lijklema (26) has suggested recently that the slipping plane might be located at a given field strength in the double layer. Several authors (89–93) have found the viscosity of a liquid to increase in high electric fields. Andrade and Dodd (89) concluded that the increase in viscosity is proportional to the square of the field strength

$$\frac{d\eta}{\eta} = fE^2 \tag{36}$$

where E is the field strength in electrostatic units and f (the "visco-electric constant") is a constant of the order of 2×10^{-7} in organic solvents. According to this relation the viscosity would increase markedly at fields of 10^6 volts per centimeter. These field strengths can be reached in the double layer. Unfortunately no reliable data exist for water, but it would certainly be worth while to work out this idea in order to relate ζ with ψ_0.

In the present situation one should be cautious in identifying potentials calculated from electrokinetic data with those derived from adsorption or other thermodynamic measurements.

The situation is least unfavorable when potentials and electrolyte content are low, so that high field strengths do not occur in the double layer.

In the electrophoresis of proteins the first condition is met with often, but the last one is not, and consequently it is uncertain whether one should expect the electrophoretic charge and the adsorption charge or titration charge to be identical.

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Chapter 2

Acid-Base Equilibria of Proteins

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Introduction

Generally speaking, proteins owe their ability to take up or give off hydrogen ions in solution to the presence of ionizable groups in their constituent amino acids. In the case of a limited number of conjugated proteins other ionizable groups may play a role, e.g. phosphate groups, porphyrin groups, but our treatment in the present chapter will be mainly concerned with unconjugated proteins. Similarly, although the binding of other ions may significantly influence the hydrogen ionization of a protein, our discussion of such bindings will be very brief. In other words we shall be concerned mainly with the interpretation of the titration or hydrogen ionization curves of proteins on the basis of properties established for amino acids and their side chains.

We do not consider it our task to review the vast literature concerned with the experimental approach to our problem. This has been treated in several excellent review articles (1–3). We are more interested in discussing the theoretical problem of how the assembling of a large number of ionizable groups in one large molecule affects their ionization constants. The problem is of course far from being solved because our present knowledge of the state of protein molecules in solution is entirely inadequate. The general approach has been to adopt a simplified "model" of the protein in solution and then to see how well it can be made to fit experimental results. With the simplest possible model of the protein, i.e. a conducting sphere charged with the net charge of the protein molecule (4), the fit to experimental titration curves is surprisingly good for a number of proteins, considering the crudeness of this model. As a matter of fact there has been a tendency in the literature to explain deviations between experiments and predictions based on this simple model, by involving factors like "swelling," "steric hindrance," etc., not provided for in the model. Such conclusions would seem to require careful consideration in each individual case.

Attention should already here be called to the experimental fact that, with a few, and highly interesting, exceptions, the incorporation of the ionizable groups into the protein molecule does not change their ionization constants so as to make their identification doubtful [see, for example, the pioneer work of Simms (5)]. The change in environment therefore does not cause deep-going changes in the thermodynamic functions of the ionization of the groups.

Before we proceed with the main subject, we shall briefly discuss some electrochemical concepts of importance for the interpretation of protein acid-base equilibria. This discussion will be found in the two following sections.

I. ELECTROSTATIC THEORY OF DILUTE SOLUTIONS OF IONS

In this section we shall give a brief discussion of the Debye-Hückel theory and its application to polyvalent ions.

As is well known, the fundamental equation in this theory is

$$\nabla^2 \varphi_e = \varkappa^2 \varphi_e \tag{1}$$

where φ_e is the average electric potential outside a selected central ion and

$$\varkappa^2 = \frac{4\pi\epsilon^2}{DkT} \sum_i z_i^2 n_i = \frac{8\pi\epsilon^2 N}{1000DkT} \cdot \frac{\Gamma}{2} \tag{2}$$

where z_i and n_i are the charge and the number per cubic centimeter of the ions of species, i, belonging to the "ion atmosphere" surrounding the ion; ϵ is the electronic charge; N, Avogadro's number; k, Boltzmann's constant; T, temperature; and D, the dielectric constant. $\Gamma/2$ is the ionic strength.

At 25°C.

$$\varkappa^2 = \frac{8.4865}{D} \cdot \frac{\Gamma}{2} \text{ Ångströms}^{-2} \tag{3}$$

We shall not attempt a detailed discussion of the approximations made in the derivation of equation (1) but only point to the fact that the case of a large central ion of low charge, surrounded by an ion atmosphere consisting of small monovalent ions, is probably the ideal one for the application of equation (1). For the rest we refer to the discussions by Onsager (6), Kirkwood (7), Fowler and Guggenheim (8), and Kirkwood and Poirier (9).

In the following treatment we shall adopt the procedure by one of the authors (10) pointing out, however, that in principle it is entirely based on Kirkwood's early papers (11, 12). The solution of equation (1) in spherical coordinates with the center of the large ion as origin is

$$\varphi_e = \sum_{n=0}^{n=\infty} \sum_{m=-n}^{m=+n} A_{mn} r^n \Theta_r^{\,n+1} P_n^{\,m}(\mu) e^{im\alpha}; \quad \varkappa^2 > 0 \tag{4}$$

where $P_n^{\,m}(\mu)$ are the associated Legendre functions of the first kind and

$$\Theta_r^{\,n+1} = \frac{1}{r}\frac{\partial}{\partial r}\left(\frac{1}{r}\frac{\partial}{\partial r}\left(\frac{1}{r}\frac{\partial}{\partial r}\left(\cdots e^{-\varkappa r}\right)\right)\right) \text{ (}n + 1\text{ differentiations)} \tag{5}$$

A_{mn} are constants to be determined by boundary conditions, n and m are integers; $\mu = \cos v$; r, v, and α are spherical coordinates. If $\varkappa = 0$, equation (1) reduces to Laplace's equation

$$\nabla^2 \varphi_i = 0 \tag{6}$$

the solution of which is of a similar form as equation (4), viz.,

$$\varphi_i = \sum_{n=0}^{n=\infty} \sum_{m=-n}^{m=+n} B_{mn} r^n P_n^{\,m}(\mu) e^{im\alpha}; \quad r < 1 \tag{7}$$

or a corresponding form for $r > 1$ containing a series in r^{-n-1}.

We shall now consider the system diagrammed in Fig. 1.

The spherical ion with radius b has a number S_0 of electric point charges at fixed sites. The distance a from the center measures the closest approach of the center of an ion belonging to the ion atmosphere. Introducing that both the potential and the normal component of the displacement vector vary continuously across the boundaries, a series of equations is obtained permitting the evaluation of the constants A_{nm}, B_{nm}, etc., and of φ_i, φ_x, and φ_e.

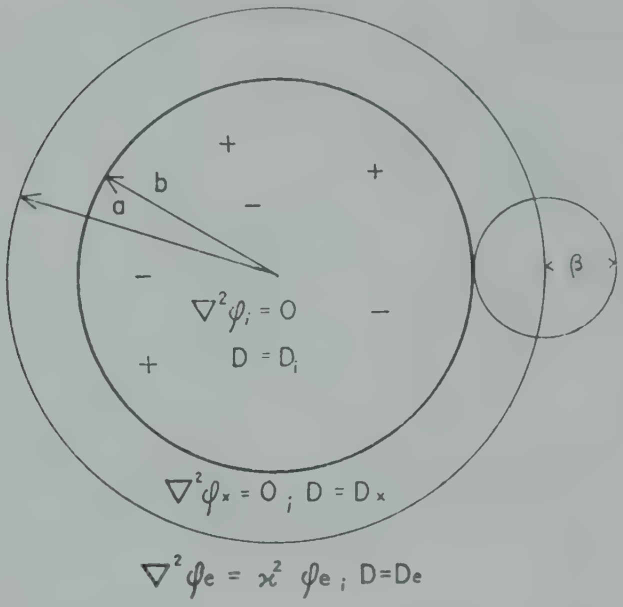

FIG. 1. Spherical ion system; solvent with ion atmosphere.

The potential which interests us here is φ_i. Putting $D_x = D_e$ for the sake of simplicity, we get

$$\varphi_i = \sum_s \frac{e_s}{D_i r_{rs}} - \frac{1}{D_i} \sum_{n=0}^{n=\infty} \sum_{m=-n}^{m=n} \sum_s e_s \frac{(n-|m|)!}{(n+|m|)!} \frac{r^n r_s^n}{b^{2n+1}}$$
$$(1 + L_n) P_n^m(\mu) P_n^m(\mu_s) e^{im(\alpha - \alpha_s)} \quad (8)$$

at a point with coordinates r, v, and α; r_{rs} is the distance from this point to charge e_s. L_n is given by

$$L_n = \frac{(2n+1)\left\{2n + 1 + \left(1 - \frac{a}{b}\right)^{2n+1} T_{n+1}\right\}}{(2n+1)n(\sigma - 1) + \left\{(\sigma(n+1) + n)\left(\frac{a}{b}\right)^{2n+1} + n(\sigma - 1)\right\} T_{n+1}} \quad (9)$$

where
$$\sigma = \frac{D_e}{D_i} \quad (10)$$

and
$$T_{n+1} = a^2 \frac{\Theta_a^{\,n+2}}{\Theta_a^{\,n+1}} \quad (11)$$

is a function of κa which approaches $-2n - 1$ when κ approaches 0 and is related to Kirkwood's $K_n(\kappa a)$ [cf. reference (11)] by the expression

$$T_{n+1} = -(2n+1) \frac{K_{n+1}(\kappa a)}{K_n(\kappa a)}$$

In order to determine the "electric free energy" ψ of the ion in the given solvent we use the Güntelberg-Müller charging process

$$\psi = \sum \int_0^{e_s} (\varphi_i)_s \, de_s \tag{12}$$

and obtain with some rearrangements and simplifications

$$\psi = \frac{1}{2D_i} \sum_{s=1}^{S_0} \frac{e_s^2}{\omega_s} + \frac{1}{2D_i} \sum_{s}^{S_0} \sum_{\substack{p \\ s \neq p}}^{S_0} \frac{e_s e_p}{r_{sp}} - \frac{1}{2D_i} \sum_{n=0}^{\infty} \sum_{s}^{S_0} \sum_{p}^{S_0} e_s e_p \frac{r_s^n r_p^n}{b^{2n+1}} (1 + L_n) P_n(\mu_{sp}) \tag{13}$$

where $\omega_s = 0$ is the "radius" of the point charge e_s, r_{sp} the distance between charges e_s and e_p, and μ_{sp} the cosine of the angle between r_s and r_p. The first term on the right side of equation (13), the self-energies of the point charges, will cancel in all of the following operations and will be neglected. By definition

$$\ln \gamma = \frac{\psi - \psi_0}{kT} \tag{14}$$

where γ is the activity coefficient and ψ_0 the free electric energy at infinite dilution $\varkappa = 0$. From equations (14) and (13) we get

$$\psi = \psi_0 + kT \ln \gamma \tag{15}$$

where ψ_0 is given by

$$\psi_0 = -\frac{1}{2D_i} \sum_{n=0}^{\infty} \sum_{s=1}^{S_0} e_s^2 \frac{r_s^{2n}}{b^{2n+1}} \frac{(n+1)(\sigma-1)}{\sigma(n+1)+n} + \frac{1}{2D_i} \left[\sum_{\substack{s=1 \\ s \neq p}}^{S_0} \sum_{p=1}^{S_0} \frac{e_s e_p}{r_{sp}} - \sum_{n=0}^{\infty} \sum_{\substack{s=1 \\ s \neq p}}^{S_0} \sum_{p=1}^{S_0} e_s e_p \frac{r_s^n r_p^n}{b^{2n+1}} \frac{(n+1)(\sigma-1)}{\sigma(n+1)+n} P_n(\mu_{sp}) \right] \tag{16}$$

and $\ln \gamma$ by

$$\ln \gamma = -\frac{1}{2D_e kT} \sum_{n=0}^{\infty} \sum_{S=1}^{S_0} \sum_{p=1}^{S_0} e_s e_p \frac{r_s^n r_p^n}{a^{2n+1}} \frac{(2n+1)\sigma^2 B_n P_n(\mu_{sp})}{(\sigma(n+1)+n)^2 \left\{1 + \frac{B_n}{2n+1} \left(\frac{n(\sigma-1)}{\sigma(n+1)+n} \left(\frac{b}{a}\right)^{2n+1} + 1 \right)\right\}} \tag{17}$$

where $B_n = -T_{n+1} - (2n+1)$.

Table I gives some values for B_n.

TABLE I

VALUES FOR B_n

n	B_n
0	$\varkappa a$
1	$\frac{\varkappa^2 a^2}{1 + \varkappa a}$
2	$\frac{\varkappa^2 a^2 + \varkappa^3 a^3}{3(1 + \varkappa a) + \varkappa^2 a^2}$

The equations (16) and (17) are identical with those of Kirkwood (11). The first term on the right side of equation (16) comprises all the finite self-energy terms originating in the interaction of each charge with the field it creates in the spherical region with $D_i \neq D_e$ ($\varkappa = 0$). If $D_i = D_e$, $\sigma = 1$ or if $b = \infty$, the term vanishes. The transfer work of bringing a charge from one spherical region to another is given by

$$\Delta\psi_0 = \frac{\epsilon^2}{2D_i} \sum_{n=0}^{\infty} \frac{(n+1)(\sigma - 1)}{\sigma(n+1) + n} \left[\left(\frac{r_s^{2n}}{b^{2n+1}} \right)_1 - \left(\frac{r_s^{2n}}{b^{2n+1}} \right)_2 \right] \tag{18}$$

and might be of considerable magnitude (see Section IV, Fig. 3). The last two terms in equation (16) represent the point charge interaction energies at infinite dilution ($\varkappa = 0$), the summation being extended over $S_0(S_0 - 1)$ pairs of charges. The form of equation (16) is independent of the special assumptions underlying the Debye-Hückel theory but is of course intimately connected with the adopted electrostatic model which treats ions in solution as charged bodies suspended in a dielectric continuum.

Equation (17) is the general expression for the activity coefficient of a multipolar spherical ion according to the Debye-Hückel theory. It should be noted that if the sphere has s charges in the center or evenly "smeared out" on the surface, the only finite term in equation (17) is that for $n = 0$. We therefore get

$$\ln \gamma = -\frac{1}{2D_e kT} \sum_s \sum_p e_s e_p \frac{1}{a} \frac{\sigma^2 B_0}{\sigma^2(1 + B_0)} = -\frac{s^2 \epsilon^2}{2D_e kT} \frac{\varkappa}{1 + \varkappa a} \tag{19}$$

Equation (19) is the well-known Debye-Hückel expression for single ions with valency s.

It should finally be noted that all terms in equations (16) and (17) are invariant to the change of sign of all charges. Thus the ion considered and its "inverse" with respect to sign have identical activity coefficients.

This result, which is not necessarily valid for actual ions, is a consequence of our simple electrostatic model of ions in solution. In particular the distance a of closest approach is assumed to be the same for anions and cations of the ion atmosphere, and the solvent itself is regarded as a continuous dielectricum.

For corresponding calculations of ψ_0 for ions shaped as prolate ellipsoids we refer to reference (10).

II. SIMPLE ACID-BASE EQUILIBRIA. pH

The fundamental equation in proton equilibria in solution is the following

$$A \rightleftharpoons B + H^+ \tag{20}$$

where the acid, A (e.g. $RCOOH$ or RNH_3^+), and the base, B (e.g. $RCOO^-$ or RNH_2), are said to be mutually corresponding [Brønsted (13)]. Since ions are involved the equilibrium condition should be written

$$\bar{\mu}_A = \bar{\mu}_B + \bar{\mu}_H \tag{21}$$

where $\bar{\mu}_A$, $\bar{\mu}_B$ and $\bar{\mu}_H$ are the electrochemical potentials of the three components [see, for example, Guggenheim (14)]. These potentials are formally connected with the chemical potentials through the expressions

$$\bar{\mu}_A = \mu_A + \epsilon_A \cdot \psi \text{ etc.} \tag{22}$$

where ψ is the electric potential in the solution and ϵ_A is the molecular charge of the component A. Owing to the fact that the sum of the molecular charges is the same on both sides of (20), the electric terms will cancel in a homogeneous phase and equation (21) may be replaced by

$$\mu_A = \mu_B + \mu_H \tag{23}$$

remembering, however, that only chemical potentials of uncharged components or certain linear combinations of chemical potentials of charged components have a strict physical meaning (14).

We now introduce the relations:

$$\begin{aligned} \mu_A &= \mu_A{}^\circ + RT \ln a_A = \mu_A{}^\circ + RT \ln m_A\gamma_A \\ \mu_B &= \mu_B{}^\circ + RT \ln a_B = \mu_B{}^\circ + RT \ln m_B\gamma_B \\ \mu_H &= \mu_H{}^\circ + RT \ln a_H = \mu_H{}^\circ + RT \ln m_H\gamma_H \end{aligned} \tag{24}$$

where the activities a_A, a_B, and a_H are so defined that they approach the corresponding molal concentrations m at infinite dilution (the activity coefficients, γ, approach 1). We obtain from (23)

$$RT \ln K = \mu_A{}^\circ - \mu_B{}^\circ - \mu_H{}^\circ = RT \ln \frac{a_B \cdot a_H}{a_A} \tag{25}$$

where K, the "complete" thermodynamic acid dissociation constant, is a true unambiguously defined constant. The activities and activity coefficients of individual ions have again no physical meaning but may be used formally in the description of the ionization.

The activity coefficients introduced take care of the depression of the activities due to interionic forces and to all other associations than the molecular event $B + H^+ \rightarrow A$. At high dilution they are supposed to vary according to the limiting law of Debye-Hückel (see below) and at medium concentrations to some half-empirical law valid for strong electrolytes. Thus "bound" hydrogen ions are included in A, "free" hydrogen ions in H^+. In addition to γ we may define a set of stoichiometric activity coefficients f_i which like γ_i approach 1 at infinite dilution but which reflect not only the interionic forces but also all molecular associations, e.g. $Y + Cl^- \rightarrow YCl^-$. Denoting the total concentration of "free" and "bound" Cl^- by $m_{Cl}°$

$$a_{Cl} = m_{Cl}°f_{Cl} = m_{Cl}\gamma_{Cl} \tag{26}$$

We shall return to this question below.

The measurement of K may be accomplished in cells "without liquid junction," i.e., containing only one homogeneous liquid phase, by combining μ_H with the potential of a monovalent negative ion. Thus if A is uncharged, $\mu_B + \mu_H$ may be measured unambiguously. In general, however, μ_H is combined with the potential of a negative ion for which a good reversible electrode is available, e.g. Cl^-. Numerous measurements [cf. references (15–17)], have been carried out with the cell

$$H_2, Pt \,|H^+, A, B, X^+, Cl^-|\, AgCl, Ag \tag{I}$$

where X^+ is the counterion of Cl^- and the counterions of A and B have been omitted. By measuring the electromotive force of this cell at different concentrations of A and B (and HCl and XCl) results are obtained which may be utilized in the calculation of K.

If the concentration of XCl is large in proportion to A, B, and H^+, a comparison of the electromotive force of the above cell (E) with that (E_x) of a similar cell containing only HCl and XCl ($m_{HCl} \ll m_{XCl}$) will directly render the value of m_H:

$$F(E_x - E) = RT \ln \frac{a_H a_{Cl}}{(a_H a_{Cl})_x} = RT \ln \frac{m_H}{(m_H)_x} \tag{27}$$

assuming that m_{XCl} is the same in both cells so that γ_H, γ_{Cl} and m_{Cl} cancel. With known concentrations of A and B the so called concentration constant K_C is directly obtained

$$K_C = K \frac{\gamma_A}{\gamma_B \gamma_H} = \frac{m_B m_H}{m_A} \tag{28}$$

K_C is just as well defined as K, which it approaches with increasing dilution. An extrapolation to K is therefore possible on the basis of a series of measurements with falling salt concentration.

Naturally the above experiments are not the only ones which will lead to K. In general the electromotive force of cell (I) with known concentration of Cl^- determines the quantity $m_H\gamma_H\gamma_{Cl}$, i.e.

$$F(E_0 - E) = \mathrm{R}T \ln m_H\gamma_H\gamma_{Cl} + \mathrm{R}T \ln m_{Cl} \tag{29}$$

where E_0 is the electromotive force, when $m_H m_{Cl}\gamma_H\gamma_{Cl} = 1$. E_0 is obtained from measurements of cells with known values of $m_H m_{Cl}\gamma_H\gamma_{Cl}$, e.g. solutions of HCl (with or without XCl). According to Güntelberg (18) $E_0 = 0.2255_0$ volt at 20°C.

In analogy with Sørensen's original suggestion in 1909 (19) a quantity pwH (see Bates (17)) may be defined by

$$\mathrm{p}w\mathrm{H} = -\log m_H\gamma_H\gamma_{Cl} = \frac{F \log e}{\mathrm{R}T}(E - E_0) + \log m_{Cl} \tag{30}$$

and equation (25) may be transformed into

$$\mathrm{p}K = \mathrm{p}w\mathrm{H} + \log \frac{m_A}{m_B} + \log \frac{\gamma_A\gamma_{Cl}}{\gamma_B} \tag{31}$$

where pK = $-\log K$ (Bjerrum Ref. 25). The last term in equation (31) may be determined unambiguously.

Turning now from the measurement of K in a simple system like that considered above to the general problem of acidity determination in arbitrary solutions of insufficiently characterized composition (protein solution, biological fluids, etc.) pwH has the unquestionable advantage as a *measure of acidity* that, like K or pK, it is a thermodynamic function which undergoes meaningful variations with temperature, pressure, and solvent. Its disadvantages are, however, equally obvious. Thus in a system of unknown composition γ_{Cl} in equation (30) must be replaced by the stoichiometric activity coefficient f_{Cl}, discussed above. From equation (26) it is seen that pwH may deviate appreciably more than $\log \gamma_{Cl}$ from $-\log m_H\gamma_H$ if Cl^- is bound by components of the system. Although this deviation may be reduced by adding a large excess of chloride over that required to saturate the "binding sites," the fact remains that pwH is unsuitable as a general acidity measure.

Naturally the quantity

$$\mathrm{pHCl} = -\log a_H a_{Cl} \tag{32}$$

may be used in characterizing the binding of HCl to components of the system.

In addition to this theoretical difficulty a more practical one should be mentioned, i.e. the silver-silver chloride electrode (and other reversible electrodes of this type) is very sensitive to influences of various kinds (oxidation, reactions with thiol groups, etc.) which may play a considerable role in acidity measurements in protein solutions.

Therefore, it is natural that the majority of acidity determinations in the past fifty years have been made with hydrogen ion electrodes alone, of which in our time there are two robust types, the hydrogen electrode (mentioned above) and the glass electrode. The quantity pH, whose definition has followed a rather irregular path since it was first suggested by Sørensen, is most adequately defined by the potential E_{12} of the following cell

$$\mathrm{H_2, Pt}\ |\text{solution 1}|\ \begin{matrix}\text{sat.}\\ \text{KCl}\\ \text{solution}\end{matrix}\ |\text{solution 2}|\ \mathrm{H_2, Pt} \tag{II}$$

$$\mathrm{pH}_2 - \mathrm{pH}_1 = \frac{FE_{12}\log e}{\mathrm{R}T} \tag{33}$$

As is well known, the bridge of saturated KCl solution serves to minimize the diffusion potential between solution 1 and solution 2. Since no experiments can be devised for measuring these diffusion potentials exactly for arbitrary solutions, $-\log\,[(a_{\mathrm{H}})_1/(a_{\mathrm{H}})_2]$ cannot be derived in an exact way from measurements with cell II and cannot be identified with $\mathrm{pH}_2 - \mathrm{pH}_1$.

According to Guggenheim (20) the best one can say is that pH is equal to the quantity $-\log m_{\mathrm{H}}\gamma_{\mathrm{H}}^{?}$ where $\gamma_{\mathrm{H}}^{?}$ is a miserable coefficient which has no simple physical meaning and is more complicated than the activity coefficients of electrolytes because it depends to an unknown extent upon conditions in the diffusion zones. It does however approach 1 at infinite dilution, and in solutions of known composition and finite concentrations it is found to be approximately proportional to γ_{H} calculated theoretically on the basis of reasonable extrathermodynamical assumptions (Debye-Hückel theory). We shall therefore choose, for practical reasons, to adopt pH as a measure of acidity and follow a generally accepted procedure (17) in defining it by means of cell II in which, for example, the pH of solution 2, the standard buffer solution, is established through measurement of pwH and reasonable theoretical estimates of γ_{Cl}. (Naturally in routine measurements the standard half-cell in II may be replaced by a calomel half-cell; the hydrogen electrode in solution 1, by a glass electrode.) We refer the reader to Bates's excellent book (17).

Returning now to equation (25) we may in analogy to (31) write

$$\mathrm{p}K^{?} = \mathrm{pH} + \log\frac{m_{\mathrm{A}}}{m_{\mathrm{B}}} + \log\frac{\gamma_{\mathrm{A}}}{\gamma_{\mathrm{B}}} \tag{34}$$

where γ_A/γ_B is defined by suitable extrathermodynamical assumptions. [γ_A/γ_B cannot be measured exactly because the difference in charge ($\epsilon_A - \epsilon_B$) between A and B is always +1.] The difference

$$pK - pK^? = \log \frac{\gamma_A}{\gamma_B \gamma_H} - \log \frac{\gamma_A}{\gamma_B} + \log \gamma_H{}^? = \log \gamma_H{}^? - \log \gamma_H$$

is a function only of the diffusion potential between solution 1 and the saturated KCl solution in cell II. It is usually small.

Drawing the last term in equation (34) into $pK^?$ we get

$$pK' = pH + \log \frac{m_A}{m_B} \tag{35}$$

where $K' = 10^{-pK'}$ is the so called *incomplete* ionization constant, the measurement of which is the simplest possible, i.e. involving only a dissolution of equal molar quantities of A and B and a determination of the pH of the solution. In certain cases rather precise values of pK may be obtained from pK' on the basis of reasonable theoretical values of γ_A/γ_B. If A or B is uncharged, we have according to the Debye-Hückel limiting law the low value

$$\log \frac{\gamma_A}{\gamma_B} \sim \pm 0.5\sqrt{\Gamma/2}$$

where $\Gamma/2$ is the ionic strength. If however A has n positive charges and B consequently $n - 1$, we should theoretically find

$$\log \frac{\gamma_A}{\gamma_B} \sim -(n^2 - (n-1)^2)0.5\sqrt{\Gamma/2}$$

a value which may become uncomfortably high and uncertain. Extrapolation of pK' to infinite dilution yields pK values with an error that has been estimated to be of the order of 0.02 pH units in the pH interval between 2 and 12 (17).

Considering finally pH measurements in protein solutions during titration with strong acids or bases the experimental set up is generally so chosen that the ionic environment of the macromolecules does not change appreciably within the pH range covered. This is done to reduce the serious difficulties in the theoretical treatment to a minimum. The protein concentration is made as low as possible and a mono-monovalent salt like KCl is added to give ionic strengths of about 0.1 and more. The contribution of the protein ions to the ionic strength may then be neglected with greater justification.

Under these circumstances $\gamma_H{}^?$ may be considered approximately constant, and what is measured with the hydrogen ion electrode is essentially the variation of m_H during titration:

$$\Delta\text{pH} = \log \frac{(m_{\text{H}})_1}{(m_{\text{H}})_2} \tag{36}$$

Since, however, the theoretical treatment operates with activity coefficients of single ions and describes ionization equilibria in such terms, the reader will find in the following sections repeated references to the equation

$$\text{p}K = \text{pH} + \log \frac{m_{\text{A}}}{m_{\text{B}}} + \log \frac{\gamma_{\text{A}}}{\gamma_{\text{B}}} \tag{37}$$

which formally coincides with equation (25) putting

$$\text{p}a_{\text{H}} = -\log a_{\text{H}} \sim \text{pH} \tag{38}$$

In equation (37) the activity coefficients should be calculated from available theories of interionic forces.

III. The Acidic and Basic Groups of Proteins

As stated in the introduction, we shall confine ourselves to unconjugated proteins. Some of the considerations on the following pages may, however, easily be extended also to conjugated proteins. So far, somewhat more than twenty amino acids have been isolated from protein hydrolyzates. Of these only the ones with acidic or basic side groups (including cystine) may contribute more than two ionizable groups per molecule of polypeptide. Consequently, from the point of view of the acid-base equilibria of proteins, it is the amino acids with acidic or basic side groups that are of major interest. In Tables II and III the acid dissociation constants (pK or pK') of these amino acids in water at 25°C. are listed together with the dissociation constants of other amino acids, some peptides, and derivatives for comparison.

TABLE II

Thermodynamic Functions for Amino Acids and Peptides at 25°C.

Substance	pK_1 COOH	pK_2' NH_3^+	$\Delta H_1°$ (cal./mole)	$\Delta H_2°$ (cal./mole)	Reference
Gly	2.350	9.778	1156	10806	(67)
Ala	2.348	9.867	773	10980	(68)
Leu	2.329	9.747	382	10814	(68)
Asp	1.995 (α)	10.006	1783 (α)	9025	(69)
	3.910 (β)		1110 (β)		
Arg	1.807	9.01			(70)
Gly-Gly	3.140	8.252	+32	10600	(69, 71)
Gly-Ala	3.153		−563		(71)
Gly-Leu	3.180		−752		(71)
Gly-Ser	2.981		+189		(71)

TABLE III

pK' VALUES AT 25°C.

Substance	pK_1'	pK_2'	$\Delta H_1^{\circ\prime}$	$\Delta H_2^{\circ\prime}$	Reference
Glu	2.16 (α) 4.32 (γ)	9.96	1900 (α) 1040 (γ)	11200	(72)
Tyr	2.20	9.11 (NH_3) 10.07 (OH)			(72)
His	1.82	9.17 (NH_3) 6.00 (Im.)	1200	9400 (NH_3) 6900 (Im.)[a]	(72)
Lys	2.18	8.95 (α) 10.53 (ϵ)	300	11600 (α) 11350 (ϵ)	(72)
Gly	2.34	9.60			(72)
Glycinamide		7.93			(72)
Glycine ethyl ester		7.73			(72)
Gly-Gly	3.08	8.26			(72)
Gly-Gly-Gly	3.26	7.91			(72)
Gly-Gly-Gly-Gly	3.05	7.75			(72)
Ala-Ala (LL)	3.30	8.14			(73)
Ala-Ala (LD)	3.12	8.30			(73)

[a] Imidazole.

Equation (24) in Section II defines the quantities μ_A°, μ_B° and μ_H° as being the chemical potentials of the components when a_A, a_B and a_H are all unity (the "ideal one-molal standard state"). It is conventional to write

$$-\Delta F^\circ = \mu_A^\circ - \mu_B^\circ - \mu_H^\circ = RT \ln K = -2.3026RT \cdot pK \qquad (39)$$

where ΔF° is termed the "free energy" change per mole of the process [equation (20)] from left to right under the standard conditions. In the following we will always think of these as aqueous solutions. K, pK, and ΔF° may be similarly defined in other amphiprotic solvents.

$$\mu_A^\circ = \left(\frac{\partial F}{\partial n_A}\right)_{p,T,n} \text{etc.} \qquad (40)$$

where n_A is the number of moles of A in the system in the standard state, and

$$F = H - TS \qquad (41)$$

Furthermore, it is conventional to write for the standard state

$$\Delta H^\circ = \left(\frac{\partial H}{\partial n_A}\right)_{p,T,n} - \left(\frac{\partial H}{\partial n_B}\right)_{p,T,n} - \left(\frac{\partial H}{\partial n_H}\right)_{p,T,n} = RT^2\left(\frac{\partial \ln K}{\partial T}\right)_p \quad (42)$$

and a similar expression for ΔS° so that

$$\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ \quad (43)$$

where

$$\Delta S^\circ = -\left(\frac{\partial(\Delta F^\circ)}{\partial T}\right)_{p,n} \quad (44)$$

The majority of measurements on amino acids and peptides has been made with cells of type (II) containing a salt bridge. Some of the values obtained for the incomplete dissociation constants (pK') are listed in Table III. For some amino acids and peptides both pK (Table II) and pK' values have been reported. A comparison between corresponding values in Tables II and III gives an idea of how small increments in pK' values, e.g. for carboxyl groups in different amino acids, may be considered significant. Tables II and III also list some values of ΔH° and $\Delta H^{\circ\prime}$. $\Delta H^{\circ\prime}$ is given by equation (42) when K' is substituted for K. The difference in $\Delta H^{\circ\prime}$ values for carboxyl, imidazole, and amino groups should be noted. This difference may be used in the identification of the ionizable groups contributing to various parts of a protein titration curve as demonstrated by Wyman (21) for oxyhemoglobin. Table IV lists some assigned intrinsic pK values for "typical" ionizable groups in proteins (see Section VII). As mentioned in the introduction, the incorporation of an ionizable group into a protein molecule usually (with some interesting exceptions) changes the intrinsic pK of the group to a moderate degree (less than one unit) compared with that of the same group in a small peptide (Table IV).

The theoretical treatments of protein acid-base equilibria, discussed in Section VI, attempt to construct the titration curve of a given protein on the basis of its amino acid composition and some model of its structure in solution. This problem reduces to accounting for the various interactions between a protein ionizable group in its acid and base form and the rest of the protein molecule. In other words, determining the pK difference between a given ionizable group in a protein molecule of known structure and the same group in a small peptide. Formally the protein may be considered to be substituted in the amino acid carrying the ionizable group under consideration, and we shall therefore briefly discuss the general effect of a substituent on the pK of an acid group.

From theoretical considerations and based on extensive measurements of acid dissociation constants found in the literature, such an effect may be described as (see e.g. 22–24) (a) electrostatic, (b) inductive, (c) mesomeric or hyperconjugative, (d) local[1] (hydrogen bonding, steric effect, etc.

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[1] Relative to the substituent.

TABLE IV

Intrinsic Dissociation Constants pK^* at Approximately 25°C. from Analysis of Titration Curves

	α-COOH	β, γ-COOH	Imidazole	α-NH_2	ϵ-NH_2	Phenoxyl	SH	Guanidyl
Ovalbumin (89)		4.29	6.7–6.8		10.07			
β-Lactoglobulin (91)		4.60	6.7–6.8					
Ribonuclease (103)	(3.75)	4.0; 4.7	6.5	7.8	10.2	9.95		> 12
Bovine serum albumin (108)	(3.75)	3.9–4.0	7.0	7.8	9.8	10.35		> 12
Peptides (1 after, 29)	3.0–3.2	3.0–4.7	5.6–7.0	7.6–8.4	9.4–10.6	9.8–10.4	8.5–9.1	11.6–12.6

Our reason for listing these effects is, by separate consideration of each one of them, to try to estimate their relative importance in influencing the pK of a protein acidic group. Our present knowledge of protein structure in solution is in general insufficient for such an estimation, and the conclusions drawn can only be considered as suggestive for the construction of models to interpret the acid-base equilibria of proteins.

1. The ratio of the two dissociation constants of some organic dicarboxylic acids can be quantitatively accounted for by simple electrostatic theory (25). The strength of an acid group situated in a protein molecule is therefore expected to show a strong dependence on the net charge of the molecule as well as on the charge and dipole distribution in the immediate vicinity of the acid group. Furthermore, the size and shape of the protein molecule must be of importance as it constitutes a region of low dielectric constant (26).

2. The inductive or electron-displacing effect is relayed through chemical bonds. It is a medium range effect (22), being transmitted only through a few bonds. Its influence on an acid group is therefore not expected to change much from simple peptides to proteins. No sharp distinction exists between the inductive and the electrostatic effect.

3. The mesomeric effect, not counting its contribution to the acid strength of the groups proper, like carboxyl, hydroxyphenyl, guanidinium, is believed to play a minor role in fixing the pK of a protein acidic group. The reason is that in simple peptides the π orbitals of the acidic group are separated from other π orbitals by a single-bonded carbon atom.

The hyperconjugative effect, although weak, may possibly influence the pK of protein acidic groups with resonance, like carboxyl and imidazolium, namely if the hyperconjugation is prevented by twisting of the acidic group by a neighboring group.

4. Local effects influence the pK of simple acids in various ways. Thus *o*-methyl anilinium ion ($K = 4.1 \times 10^{-5}$) at 25°C. is a stronger acid than anilinium ion ($K = 2.4 \times 10^{-5}$). The effect of the methyl group is presumably "steric" (22). Differential depression of the hydration of the charged and uncharged amino group in the acid and base may account for the observed change in K on methyl ortho-substitution.

The effect of intramolecular hydrogen bonding on the strength of acids, like *o*-salicylic and maleic, is well known, but also some dicarboxylic acids with apparent greater "flexibility" show strong hydrogen bonding, e.g. diethylmalonic acid and tetramethylsuccinic acid (27).

It should be emphasized that the role of local effects (steric, hydrogen bonding, as well as others more sophisticated) depends on the structure of the protein molecule to a much higher degree than do any of the other effects listed. Under proper conditions both steric (3) and hydrogen-bonding (28) effects are expected significantly to influence the strength of some protein acidic groups (see Section VII).

Summarizing, we may say that any molecular protein model, attempting to describe the acid-base equilibria of a given protein, will have to incorporate the electrostatic interaction of charges on a single protein molecule. Furthermore, an electrostatic model may not be sufficient, depending on the structure of the protein molecules at hand, to interpret the acid-base equilibria of the protein solution.

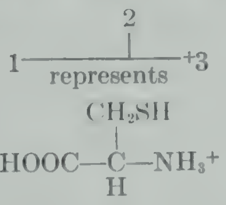
In the following section we shall critically examine the electrostatic effect in simple organic molecules and its evaluation in protein models.

TABLE V

MICROSCOPIC pK' VALUES OF CYSTEINE AT 23°C., IONIC STRENGTH 0.15 (30)

Carboxyl		Sulfhydryl		Ammonium	
pK'_1	1.71	pK'_2	7.45	pK'_3	6.77
pK'_{21}	2.79	pK'_{12}	8.53	pK'_{13}	8.86
pK'_{31}	3.80	pK'_{32}	9.09	pK'_{23}	8.41
pK'_{231}	4.74	pK'_{132}	10.03	pK'_{123}	10.36

Before ending this section, however, we shall discuss the ionization of cysteine as an interesting example. According to Benesch and Benesch (29) and Edsall (30) the pK' values in Table V may be given where the symbol



$$1\overset{2}{\text{———}|\text{———}}+3 \quad \text{represents} \quad \begin{array}{c}\mathrm{CH_2SH}\\ | \\ \mathrm{HOOC{-}C{-}NH_3^+}\\ \mathrm{H}\end{array}.$$

16.11.59

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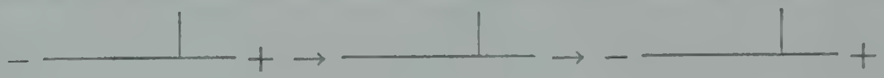
The three acidic groups situated in the same molecule are numbered 1, 2, and 3. pK_{123} = pK_{213} is the pK value of group 3 (NH_3^+) when 1 and 2 are both ionized, etc. The introduction of a negative charge on the thiol group makes both the carboxyl and the ammonium group weaker acids, which is compatible with an electrostatic effect. The difference between the ΔpK' values in the former and the latter case may here be explained by the smaller average distance between S^- and NH_3^+ as compared to that between S^- and COOH.

Several of the molecules in Table V are practically nonexistent in aqueous solution at any pH. But pK_{13}, pK_{123}, pK_{12}, pK_{132}, and pK_1 can be directly measured with cysteine, and pK_3, pK_{32}, pK_2, and pK_{23} may be obtained from measurements with cysteine ethyl ester (30), assuming that the inductive and electrostatic effects of COOH and $COOC_2H_5$ are identical. The other pK values may be obtained from relations of the type

$$\mathrm{p}K_{31} = \mathrm{p}K_{13} + \mathrm{p}K_1 - \mathrm{p}K_3 \tag{45}$$

expressing (in this particular case) that the free energy of the process



is zero.

It is evident that the influence of, for example, COO^- upon the ionization of SH must be identical with that of S^- upon the ionization of COOH, i.e.

$$\mathrm{p}K_{21} - \mathrm{p}K_1 = \mathrm{p}K_{12} - \mathrm{p}K_2 = 1.08$$

(see Table V). Similarly, it follows from equation (45) that the ammonium group makes the COOH group just as much stronger as the carboxylate group makes the ammonium group weaker, as an acid. These relationships are quite general for "substitutions" involving only proton displacements.

IV. Electrostatic Interaction in Polyvalent Acids

Normal symmetrical dicarboxylic acids show a ratio, $K_1/K_2 > 4$, between the first and the second dissociation constant. The ratio K_1/K_2 approaches 4 (the theoretical ratio, when the two carboxylic groups are noninteracting) with increasing length of the carbon chain. Bjerrum (25) interpreted the deviation of the ratio K_1/K_2 from 4 as due to electrostatic interaction between the two negative charges on the dicarboxylate ion. Neglecting all other effects than the electrostatic, he obtained the equation

$$kT \cdot \ln \frac{K_1}{4K_2} = \frac{\epsilon^2}{rD} \tag{46}$$

where r is the distance between the charges. Taking D as equal to $D_e = 78$, the dielectric constant of water at 25°C., Bjerrum obtained from equation (46) values of r for various dicarboxylic acids (Table VI). For the long-chain acids, suberic, adipic, and glutaric, the calculated r values are quite reasonable, lying between the maximum value for an elongated molecule and the average value, calculated for a molecule with free rotation about C—C bonds. However, the values obtained for the short-chain acids, succinic, methylmalonic, etc., are lower than the free-rotation value; methylmalonic acid even has the impossibly low r value 1.62Å. Bjerrum's calculations thus demonstrate the importance of the electrostatic interaction but obviously fail to account quantitatively for K_1/K_2 for short-chain dicarboxylic acids.

TABLE VI

Proton-Proton Distances r in Dicarboxylic Acids

			r Å[b].				K. and W. model[c]		
Acid	pK_1[a,c]	ΔpK_{1-2}[b]	max.[d]	Free rotation[e]	Bjerrum[f]	K. and W.[g]	d (Å.)	D_E[g]	Type
Oxalic	—	2.36	4.44	3.50	0.91	3.85	0.80	27	Spherical
Malonic	2.83	2.26	4.87	4.12	1.36	4.10	0.95	26	Spherical
Methylmalonic	3.05	1.89	4.87	4.12	1.62	4.05	0.75	32	Spherical
Ethylmalonic	2.99	2.05	4.87	4.12	1.50	4.10	0.90	29	Spherical
Diethylmalonic	2.21	4.48	4.87	4.12	0.69	3.75	2.20	14	Ellipsoidal
Succinic	—	0.84	6.66	4.66	3.65	5.75	—	—	Ellipsoidal
Glutaric	—	0.47	7.39	5.15	6.53	7.00	1.10	74	Ellipsoidal
Adipic	—	0.38	9.02	5.59	8.11	7.75	1.25	83	Ellipsoidal
Suberic	—	0.28	11.46	6.38	10.9	9.30	1.05	94	Ellipsoidal

[a] Values from reference (24).
[b] Values from reference (34).
[c] Values from reference (37).
[d] Maximum with unstrained bonds.
[e] Average with free rotation around C—C bonds.
[f] From equation (46). According to Bjerrum (25).
[g] From equation (47). K. and W. = Kirkwood and Westheimer (26,33).

Various modifications of the Bjerrum model have been suggested (31, 32) which attempt to extend the validity of electrostatic calculations also to short-chain dicarboxylic acids. The treatment of Kirkwood and Westheimer (26, 33, 34) has been the most successful. These authors in their pioneer work adopt the Kirkwood model (11) of polyvalent ions (discussed in Section I), describing the molecules and ions as spherical or ellipsoidal cavities of the uniform dielectric constant $D_i = 2.0$ (the dielectric constant of liquid hydrocarbon) surrounded by the uniform medium of dielectric constant $D_e = 78$. The charges on the ions are considered as point charges which in the ellipsoids are located at the foci. Starting with a reasonable value for the volume of the cavity, the distance r between protons[2] in the dicarboxylic acid is calculated from

$$2.303kT\Delta \mathrm{p}K_{1-2} \equiv kT \times \ln \frac{K_1}{4K_2} = \psi_{\mathrm{A}^{--}} - 2\psi_{\mathrm{HA}^{-}} \equiv \frac{\epsilon^2}{rD_E} \qquad (47)$$

where $\psi_{\mathrm{A}^{--}}$ and $\psi_{\mathrm{HA}^{-}}$ are the electrical free energies [given for spherical ions by equation (16)] of the divalent and monovalent ion, respectively, and D_{E} is the effective dielectric constant (Table VI). These calculations give r values which do not deviate much from those of Bjerrum for the long-chain acids, but in addition they give reasonable r values for the short-chain acids.

Thus the Kirkwood-Westheimer model ascribes the breakdown of the Bjerrum equation (46) for short-chain dicarboxylic acids to the presence of a region of low dielectric constant, connecting the two carboxyl groups. Such a model will only yield the required increase in electrostatic interaction over that calculated from equation (46), if the charges are actually *embedded* in the region of low dielectric constant. Placing the two charges outside the cavity leads to an increase in the effective dielectric constant (Fig. 2). The Kirkwood-Westheimer model has also been applied to the ionization equilibria of diamines, amino acids, and acids with polar substituents (33, 34), and it is generally accepted as the best model available for electrostatic interaction in polyvalent acids.

We shall now consider the electrostatic interaction between ionizable groups in the same molecule in more detail. The breakdown of equation (46) for short-chain acids may be attributed to an inductive effect as suggested by Bjerrum. This possibility was also considered by Kirkwood and Westheimer (26), who pointed out that formally a considerable part of the inductive effect in malonic acid and higher homologs may be accounted for by the cavity model. These authors emphasized that the cavity model neglects specific interactions with the medium, like electrostriction and

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[2] The charge separation in the cavity is considered to correspond to the proton-proton distance, in an attempt to compensate for the neglect of the carboxyl dipole (26

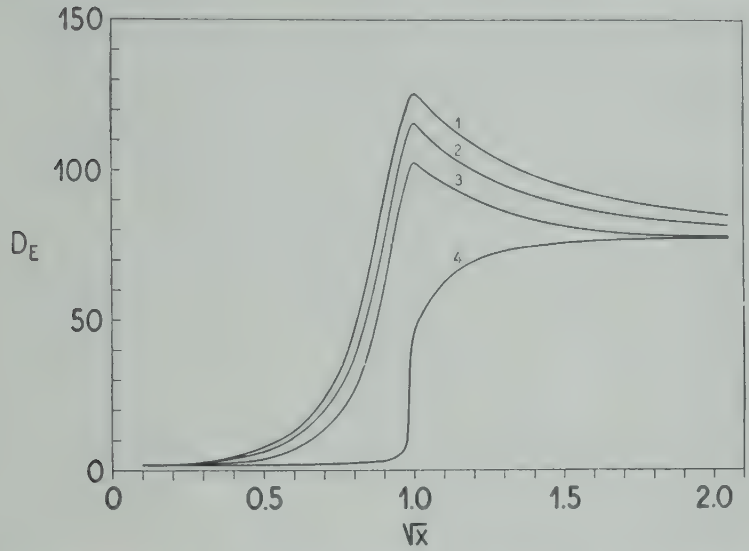

FIG. 2. The variation of the effective dielectric constant D_E of equation (47) for the interaction of two charges at distance r from the center of a spherical cavity of radius b. $\sqrt{x} = r/b$. Calculated using Fig. 1 of reference (26) and the general formulas of Section I. In curve *1*, $\cos v = -1$; in curve *2*, $\cos v = -0.5$; in curve *3*, $\cos v = 0$; and in curve *4*, $\cos v = \cos 5° = 0.996$.

dielectrical saturation, a fact which may account for the inability of the model to describe in a straightforward manner the dependence of K_1/K_2 on temperature and on the bulk dielectric constant D_e of the medium (35, 36).

As was mentioned above and illustrated by some examples in Table IV, the cavity model in general gives reasonable values for the proton-proton distance r in dicarboxylic acids. In connection with an application of the Kirkwood-Westheimer model to protein acid-base equilibria it has recently been pointed out by Tanford (37) that the smallest distance d from the charges to the cavity boundary is a crucial parameter, although it does not occur explicitly in the original equations (26, 33). Figure 2 demonstrates the rapid change in effective dielectric constant in the inner neighborhood of the cavity boundary and directly shows that a suitable effective dielectric constant may be obtained by choosing d within narrow limits. Thus all the acids in Table IV, except diethylmalonic acid, have d values close to 1.0 Å. (37).

The crucial nature of the parameter d may be offered as an explanation why in general the cavity model yields reasonable r values for short-chain dicarboxylic acids. The following example will illustrate this. For methylmalonic acid $\Delta pK_{1-2} = 1.89$ (Table VI). Assuming a spherical model with $\mu_{sp} = \cos v_{sp} = -0.33$ [see equation (16)] the following values, given in Table VI, are obtained: $r = 4.05$ Å., $d = 0.75$ Å. (34, 37). Suppose now that ΔpK_{1-2} were not equal to 1.89 but, say, $\Delta pK_{1-2} = 3.3$; assume $\cos v_{sp} =$

−0.33, and the same cavity volume as before, what would be the proton-proton distance r corresponding to $\Delta pK_{1-2} = 3.3$? The answer is $r = 3.6$ which may easily be verified from simple geometry with use of Fig. 2, and $d = 1.0$, i.e. a considerable change in ΔpK_{1-2} of $3.3 - 1.9 = 1.4$ produces only a relatively minor change in r.

Among the acids listed in Table VI diethylmalonic acid is the only one in which hydrogen-bonding in the monovalent anion strongly influences ΔpK_{1-2} (27, 38). Its r value, calculated from an ellipsoidal cavity model, is not unreasonable, lying somewhat below the free-rotation value, but its d value is by far the highest listed. This result falls in line with the example just given. Bjerrum's r values on the other hand, although too low for the short-chain acids, single out diethylmalonic acid with less than half the r value of methylmalonic or ethylmalonic acid. The cavity model is thus in a sense somewhat trivial when used to calculate proton-proton distances in dicarboxylic acids.

This is further demonstrated when attempts are made to calculate K_1/K_2 from available structural parameters of the molecule under consideration on the basis of the cavity model (37). In general, calculated values are obtained that are in only very qualitative agreement with experimental values. Tanford (37) has therefore proposed to extend the cavity model with an additional assumption concerning the values of d. He showed that a cavity model, e.g. for several dicarboxylic acids, will yield K_1/K_2 values in good agreement with experimental values on the basis of reasonable proton-proton distances, when d arbitrarily is equated to 1.00 Å. (compare Table VI). The corresponding cavity volumes in general differ considerably from those which Kirkwood and Westheimer obtained from Traube's rule (26, 36).

Finally we shall briefly discuss the intrinsic dissociation constants. One electrostatic effect on the pK values has not been emphasized by Kirkwood and Westheimer, although it has been tacitly included, and this is the work of the transfer of protons from one cavity to another. This transfer work has already been discussed in Section I. It depends to an extraordinary extent on the position of the charges within the cavity. Figure 3 shows the effect upon the pK value of an uncharged acidic group when it is moved from the center of an infinitely large spherical cavity with dielectric constant 2.00 to a distance r from the center of a spherical cavity with the same dielectric constant and finite radius b ($r < b$). For comparison Fig. 3 also shows the corresponding change in pK when an uncharged acidic group is moved from infinity in water to a distance r from the center of the same spherical cavity as above of radius b ($r > b$). It follows from Fig. 3 that carrying a unit charge from a point 1 Å. inside a cavity of radius 3 Å. to a point 1 Å. inside a cavity of radius 10 Å. requires a work which corresponds to a numerical change in pK of approximately 5 units. The corresponding work involved in the transfer of a charge from 1 Å. *outside* a cavity of radius 3 Å. to a position 1 Å. *outside* a cavity of radius 10 Å. corresponds to only 0.2 units.

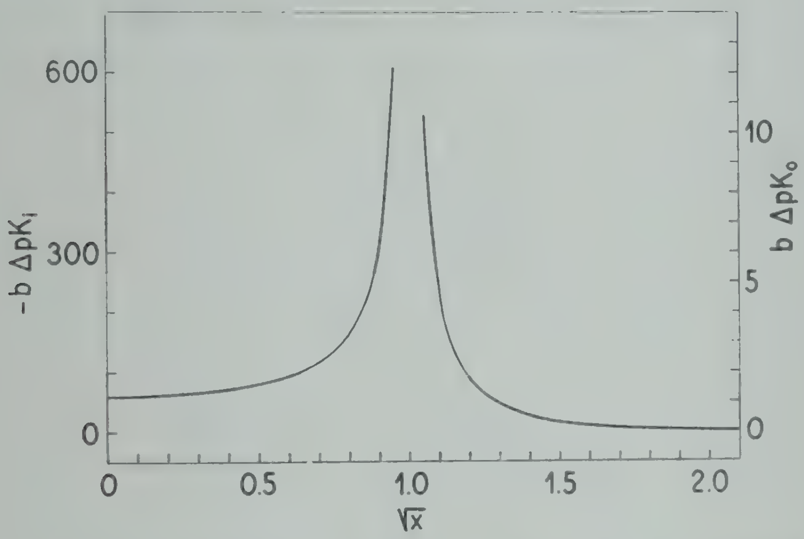

FIG. 3. *Left curve:* The change in pK of an uncharged acidic group when it is moved from the center of an infinitely large spherical cavity with dielectric constant $D = 2.00$ to a distance r from the center of a spherical cavity with $D = 2.00$ and radius b ($r < b$). *Right curve*: The corresponding change in pK when an uncharged acidic group is moved from infinity in water to a distance r from the center of a spherical cavity with $D = 2.00$ and radius b ($r > b$). $\sqrt{x} = r/b$. Calculations are based on equation (18).

The small difference between the d values of, for example, methylmalonic and ethylmalonic acid in Table VI becomes untenable, if the cavity model is used to calculate the electrostatic contribution to the first dissociation constant in the two acids. Using Fig. 3 and $\cos v_{sp} = -0.33$ for both acids (34), the data of Table VI lead to an unreasonably large difference between the first dissociation constants ΔpK_{11}.

Obviously it is not possible on the basis of the cavity model with point charges to calculate intrinsic dissociation constants, and in the literature no claim to that effect exists. Nevertheless, we want to make it clear, that when the cavity model is used to interpret acid-base equilibria of polyvalent acids, e.g. proteins, we are in general justified *only* in attaching quantitative significance to the calculated mutual interactions between charged groups but *not* to the calculated interactions leading to the intrinsic dissociation constants.

The Kirkwood-Westheimer cavity model has been the most successful of the various modifications of the simple Bjerrum model. It is therefore natural to apply it for the interpretation of acid-base equilibria of certain proteins as has recently been done (39, 40). We shall discuss this interesting application of the cavity model in Section VI, together with other electrostatic protein models that have been proposed. But already here we shall call attention to the fact that the parameter *d* preserves its crucial character also when the cavity model is used on spherical protein molecules with radius 10–20 Å. (Fig. 2). To be able to calculate the mutual electrostatic interaction between various charged groups in the protein molecule it is thus necessary, as for the simple dicarboxylic acids, arbitrarily to fix *d* within narrow limits (40). Such an arbitrary choice of *d* is of course perfectly legitimate (*d* has no simple physical meaning in itself), but with the present incomplete knowledge of protein structure and interactions between ions in water, there seems to us to be little actual advantage in using a rather complicated model which involves elaborate electrostatic calculations as well as an adjustable parameter. A more manageable protein model introducing empirical relationships between the effective dielectric constant of charge-charge interaction and charge separation may prove as useful until a better theory of electrostatic interactions in water has been formulated. Such an approach using semiempirical effective dielectric constants for nearest and next nearest neighbor interactions has been used by Rice and Harris (41) in their treatment of polyelectrolytes.

The critical examination of electrostatic interactions in polyvalent acids undertaken in this section should not lead to the impression that the whole situation of estimating such interactions is hopeless. Existing theories probably in the main lead to expressions of the right type, but the equations obtained are of a semiempirical nature and contain adjustable parameters.

V. THERMODYNAMIC TREATMENT OF PROTEIN ACID-BASE EQUILIBRIA

The treatment in this section will be quite general except for the assumptions that no associations between protein molecules occur and that all reactions considered are reversible in the pH range under consideration. Irreversible phenomena (denaturation) will be briefly considered in Section VII.

A. General Relations

The acid-base equilibria of a protein containing a total of ν uncharged acidic and basic groups per molecule may be described by the ν dissociation equilibria

$$\begin{array}{llll} H_\nu P & \rightleftarrows H_{\nu-1}P & + H^+; & K_1 \\ H_{\nu-1}P & \rightleftarrows H_{\nu-2}P & + H^+; & K_2 \\ \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot \\ HP & \rightleftarrows P & + H^+; & K_\nu \end{array} \tag{48}$$

where $H_\nu P$ is the fully protonated form of the protein and $K_1, K_2, \ldots, K_\nu$ are thermodynamic acid dissociation constants

$$K_i = \frac{a_H m_{\nu-i}\gamma_{\nu-i}}{m_{\nu-i+1}\gamma_{\nu-i+1}} \tag{49}$$

$m_{\nu-i}$ is the concentration and $\gamma_{\nu-i}$ the activity coefficient of $H_{\nu-i}P$.[2a]

In general the protein also binds ions other than H^+ as well as other solutes present, and we may formally describe the influence of such bindings on the dissociation equilibria [equation (48)] by the $\nu + 1$ composite equilibria

$$\begin{array}{lllll} H_\nu P & + I^{(\nu)} & \rightleftarrows & H_\nu P, I^{(\nu)}; & \theta_0 \\ H_{\nu-1}P & + I^{(\nu-1)} & \rightleftarrows & H_{\nu-1}P, I^{(\nu-1)}; & \theta_1 \\ \cdot & \cdot & & \cdot & \cdot \\ \cdot & \cdot & & \cdot & \cdot \\ \cdot & \cdot & & \cdot & \cdot \\ HP & + I^{(1)} & \rightleftarrows & HP, I^{(1)}; & \theta_{\nu-1} \\ P & + I^{(0)} & \rightleftarrows & P, I^{(0)}; & \theta_\nu \end{array} \tag{50}$$

where $I^{(\nu-i)}$ represents all the ions, except H^+, and other solutes bound by the protein species $H_{\nu-i}P$ carrying $\nu - i$ dissociable protons.

$$\theta_i = \frac{m'_{\nu-i}}{m_{\nu-i}} \tag{51}$$

$m'_{\nu-i} + m_{\nu-i}$ is the total concentration of protein molecules with $\nu - i$ acidic protons. $\theta_0, \theta_1, \ldots, \theta_\nu$ are functions of the composition of the medium. They all vanish if the protein combines only with protons.[3]

Equation (50) is not convenient for studying ion binding specifically, but it is well suited for the following formal discussion. For a detailed account of protein interactions with ions the reader is referred to recent comprehensive reviews (42–44).

[2a] It should be pointed out that concentrations $m_{\nu-i}$ and $m_{\nu-i+1}$ comprise a great number of different molecular species all having the same net charge but differing in the distribution of protons among proton binding sites on the protein molecule. The activity coefficients $\gamma_{\nu-i}$ and $\gamma_{\nu-i+1}$ are average values for these species. See Section VI.

[3] The term "binding" as used here is not well defined. In the following we may attribute to it any convenient definition, e.g. derived from electrophoresis studies, the remaining molecular interactions not counted in $\theta_0, \theta_1, \ldots, \theta_i$ are then included in $\gamma_0, \gamma_1, \ldots, \gamma_\nu$. When the term "ion binding" is used in the following, it refers always to binding of ions other than hydrogen ions.

Denoting the total protein concentration by m_t

$$\sum_{i=0}^{i=\nu}(m'_{\nu-i} + m_{\nu-i}) = \sum_{i=0}^{i=\nu}(1 + \theta_i)m_{\nu-i} = m_t \tag{52}$$

the $\nu + 1$ equations (49) and (52) with the unknowns $m_0, m_1, \ldots, m_\nu$ have the solutions[4] [compare Kirkwood (45)]

$$m_q = \frac{\mathfrak{D}_q}{\mathfrak{D}}$$

$$\mathfrak{D}_q = m_t a_H{}^q \frac{\gamma_0}{\gamma_q} \frac{1}{K_\nu K_{\nu-1} \cdots K_{\nu-q+1}} = m_t a_H{}^q \frac{\gamma_0}{\gamma_q} \prod_{j=0}^{j=q} K^{-1}{}_{\nu-j+1}$$

$$\begin{aligned} \mathfrak{D} &= 1 + \theta_\nu + (1 + \theta_{\nu-1}) \frac{a_H \gamma_0}{\gamma_1 K_\nu} + \cdots + (1 + \theta_0) \frac{a_H{}^\nu \gamma_0}{\gamma_\nu K_\nu K_{\nu-1} \cdots K_1} \\ &= \sum_{i=0}^{i=\nu} (1 + \theta_{\nu-i}) a_H{}^i \frac{\gamma_0}{\gamma_i} \prod_{j=0}^{j=i} K^{-1}{}_{\nu-j+1};\ K_{\nu+1} \equiv 1. \end{aligned} \tag{53}$$

The mean hydrogen ion ionization $\bar{h}$ is defined as the average number of protons dissociated per molecule from the fully protonated protein, i.e.

$$\begin{aligned} \bar{h} &= \frac{1}{m_t} \sum_{q=0}^{q=\nu} h_q(m_q' + m_q) = \frac{1}{m_t} \sum_{q=0}^{q=\nu} (\nu - q)(1 + \theta_{\nu-q}) m_q \\ &= \frac{1}{m_t \mathfrak{D}} \sum_{q=0}^{q=\nu} (\nu - q)(1 + \theta_{\nu-q}) \mathfrak{D}_q \\ &= \frac{\displaystyle\sum_{q=0}^{q=\nu} (\nu - q)(1 + \theta_{\nu-q}) a_H{}^q \frac{\gamma_0}{\gamma_q} \prod_{j=0}^{j=q} K^{-1}{}_{\nu-j+1}}{\displaystyle\sum_{i=0}^{i=\nu} (1 + \theta_{\nu-i}) a_H{}^i \frac{\gamma_0}{\gamma_i} \prod_{j=0}^{j=i} K^{-1}{}_{\nu-j+1}} \end{aligned} \tag{54}$$

If $(\theta_0, \theta_1, \ldots, \theta_\nu) = (0, 0, \ldots, 0)$, i.e. if no ion binding occurs, equation (54) reduces to the usual expression for $\bar{h}$ [see e.g. reference (1), page 474]. Equation (54) may formally be written as

$$\bar{h} = \nu - \frac{\partial \ln \mathfrak{D}}{\partial \ln a_H} \tag{55}$$

where only a_H is allowed to vary in the differentiation.

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[4] The solutions are easily obtained by computing the determinants $\mathfrak{D}_q$ and $\mathfrak{D}$ directly

Noting that

$$m_t\mathfrak{D} = \sum_{q=0}^{q=\nu} (1 + \theta_{\nu-q})\mathfrak{D}_q \tag{56}$$

and

$$q = \nu - h_q \tag{57}$$

a partial differentiation of equation (54) gives

$$\begin{aligned} \frac{\partial \bar{h}}{\partial \ln a_{\mathrm{H}}} &= \frac{\left(\sum\limits_{q=0}^{q=\nu} q(1 + \theta_{\nu-q})\mathfrak{D}_q\right)^2}{(m_t\mathfrak{D})^2} - \frac{\sum\limits_{q=0}^{q=\nu} q^2(1 + \theta_{\nu-q})\mathfrak{D}_q}{m_t\mathfrak{D}} \\ &= \left(\frac{\sum\limits_{q=0}^{q=\nu} (\nu - h_q)(1 + \theta_{\nu-q})m_q}{m_t}\right)^2 - \frac{\sum\limits_{q=0}^{q=\nu} (\nu - h_q)^2(1 + \theta_{\nu-q})m_q}{m_t} \qquad (58) \\ &= (\overline{\nu - h})^2 - \overline{(\nu - h)^2} = -\overline{(h - \bar{h})^2} = (\bar{h})^2 - \overline{h^2} \end{aligned}$$

As written, equation (58) is purely formal. We may, however, apply it to actual protein titration curves whenever the experimental conditions are such that the variation of $\gamma_0, \gamma_1, \ldots, \gamma_\nu$ and $\theta_0, \theta_1, \ldots, \theta_\nu$ with pH can be neglected. This is likely to be the case if the titration is conducted in a salt medium of ionic strength 0.1 or more. The salt added should have a common ion with the acid or base used for the titration. Under such favorable conditions equation (58) may be written

$$0.434\,\frac{\partial \bar{h}}{\partial \mathrm{pH}} \simeq -\frac{\partial \bar{h}}{\partial \ln a_{\mathrm{H}}} = \overline{h^2} - (\bar{h})^2 \tag{59}$$

thus connecting the slope of the titration curve with the standard deviation $\overline{h^2} - (\bar{h})^2$ of the hydrogen ion ionization from its mean value averaged over all molecules. Equation (59) was first derived by one of the authors several years ago [see reference (1) page 462] for proteins that were assumed to combine with protons only. Although the present derivation of equation (59) includes ion binding, it is still assumed that the protein is monodisperse.

The titration curve may be derived from equation (54). If the variation of $\gamma_0, \gamma_1, \ldots, \gamma_\nu$ with pH can be neglected, and in the absence of ion binding, the titration curve of one mole of protein is indistinguishable in form from the titration curve of a mixture of ν moles of monovalent acids with suitable dissociation constants, the titration constants $G_1, G_2, \ldots, G_\nu$ (5, 46). $\bar{h}$ may then be written[5]

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[5] $-G_1', -G_2', \ldots, -G_\nu'$ are the ν (real) roots of the equation $\mathfrak{D} = \mathfrak{D}(a_{\mathrm{H}}) = 0$. If all ionizable groups are mutually independent, the titration constants are found equal to the intrinsic dissociation constants of the various ionizable groups in the molecule.

$$\bar{h} = \sum_{i=1}^{i=\nu} h_i = \sum_{i=1}^{i=\nu} \frac{G_i}{G_i + a_{\mathrm{H}} \dfrac{\gamma_{bi}}{\gamma_{ai}}} = \sum_{i=1}^{i=\nu} \frac{G_i'}{G_i' + a_{\mathrm{H}}} \tag{60}$$

where γ_{ai} and γ_{bi} are the activity coefficients of the i'th monovalent acid and its corresponding base, respectively. γ_{ai} and γ_{bi} are likewise assumed not to vary with pH. Differentiation of equation (60) in combination with equation (59) gives

$$\frac{\partial \bar{h}}{\partial \ln a_{\mathrm{H}}} = -\sum_{i=1}^{i=\nu} \frac{G_i' a_{\mathrm{H}}}{(G_i' + a_{\mathrm{H}})^2} = -\sum_{i=1}^{i=\nu} h_i \frac{a_{\mathrm{H}}}{G_i' + a_{\mathrm{H}}} > -\bar{h} \tag{61}$$

$$0.434 \frac{\partial \bar{h}}{\partial \mathrm{pH}} \approx -\frac{\partial \bar{h}}{\partial \ln a_{\mathrm{H}}} = \overline{h^2} - (\bar{h})^2 < \bar{h} \tag{62}$$

Equation (62) is reminiscent of expressions derived in the statistical theory of fluctuations. We may, for example, apply it to a simple system. Consider a polypeptide with $2n$ carboxyl groups and n guanidyl groups and $\bar{h} = n$. What happens to $\overline{h^2} - (\bar{h})^2$ if we increase n and still maintain $\bar{h} = n$? The slope of the titration curve and consequently $\overline{h^2} - (\bar{h})^2$ will increase in the process. On the other hand

$$0 < \frac{\overline{h^2} - (\bar{h})^2}{(\bar{h})^2} = \frac{\overline{(h - \bar{h})^2}}{(\bar{h})^2} = \overline{\left(\frac{h - \bar{h}}{\bar{h}}\right)^2} < \frac{1}{\bar{h}} \rightarrow 0$$

$$n = \bar{h} \rightarrow \infty \tag{63}$$

so that what might be termed the relative standard deviation of the hydrogen ion ionization vanishes with increasing number of ionizable groups per molecule. We shall refer to this result in Section VI.

B. Isoelectric and Isoionic Points

Electrophoretic mobility measurements show that most protein molecules carry a positive charge in acid solution and a negative charge in alkaline solution. The "isoelectric point" of a pure protein may be defined operationally in terms of zero electrophoretic migration or it may be defined as the pH at which the net charge $\bar{z}$ averaged over all protein molecules is zero. From the point of view of the discussion of acid-base equilibria, the latter definition is the more convenient and we shall use it here. The two definitions may usually be considered to deviate but little from each other in terms of pH. The isoelectric point varies from protein to protein and is in addition dependent on the exact composition of the solvent medium. We may give a formal equation for the isoelectric point using equation (53). Denoting the net charge of the fully protonated species $H_\nu P$ by z^0, the net charge of $H_{\nu-i}P,I^{(\nu-i)}$ by $z^0 - i + z_{\nu-i}$ we get

$$\begin{aligned}\bar{z} &= \frac{1}{m_t}\sum_{i=0}^{i=\nu}[m_i(z^0-\nu+i)+m_i'(z^0-\nu+i+z_i)] \\ &= \frac{1}{m_t}\sum_{i=0}^{i=\nu} m_i(z^0-\nu+i)(1+\theta_{\nu-i})+\frac{1}{m_t}\sum_{i=0}^{i=\nu} m_i\theta_{\nu-i}z_i \qquad (64) \\ &= z^0-\nu+\frac{\partial \ln \mathfrak{D}}{\partial \ln a_{\mathrm{H}}}+\frac{1}{m_t}\sum_{i=0}^{i=\nu} m_i\theta_{\nu-i}z_i = z^0-\bar{h}+\frac{1}{m_t}\sum_{i=0}^{i=\nu} m_i\theta_{\nu-i}z_i = 0\end{aligned}$$

The concept of the "isoionic point" of a pure protein in a given solution was introduced by Sørensen and associates (47) as the pH at which[6]

$$z^0 - \bar{h} = 0 \qquad (65)$$

Thus in the absence of ion binding, the isoelectric and the isoionic points coincide according to our definitions. However, it follows from equation (64) that they may differ appreciably from each other if the protein combines with other ions in the solvent medium besides H$^+$. As defined, the isoionic point is a useful concept in protein titration work, but to take full advantage of it we need also to give it an operational definition. Two such definitions were implied in the original work (47).

To define the isoionic point operationally, we need isoionic protein. Such protein material is obtained, for example, after successful electrodialysis or treatment with a mixed-bed ion exchange resin. The isoionic protein produces no other noncolloidal ions when dissolved in water than H$^+$ or OH$^-$. Now, according to the *first definition* the isoionic point is the pH of the protein solution which does not change on the addition of more isoionic protein. According to the *second definition* the isoionic point is the pH of a solution of the isoionic protein in water, or in a solution of another solute which does not produce H$^+$ or OH$^-$ ions when dissolved in water alone. Thus a mixture of proteins may be isoionic. Scatchard and Black (48) have pointed out that the two definitions are conflicting, and they selected the second one for use in their study of the isoionic point of serum albumin solutions containing neutral salts. Provided the isoionic point is not too far removed from 7 and the protein concentration not too small, definition 2 can be considered to conform fairly closely to the theoretical definition [equation (65)]. Under the experimental conditions of Scatchard and Black (48) with salt concentrations ranging from 0.001 *M* and up, the second definition may in general also conform more closely to equation (65) (which was used in the interpretation of the data) than definition 1: e.g. if the protein shows strong ion-binding and if protein and strongly bound ions are present in approximately equivalent amounts, the isoionic point given by definition 1 may deviate appreciably from that given by equation (65). If a choice between definitions 1 and 2 is guided by the theoretical definition (65) the first definition may, however, be superior to the second if the isoionic point is far removed from 7 or if the protein concentration is very low. (According to definition 2 an infinitely dilute solution of any protein has the isoionic point 7.) In addition, the isoionic point of a protein dissolved in a solution of other solutes which *do* produce H^+ or OH^- ions when dissolved in water alone, is given only by definition 1.

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[6] The definition in the original paper was given as $\bar{h} = 0$ due to a different choice of zero for $\bar{h}$.

It therefore seems to us that the tendency in the literature (2, 49) to define the isoionic point operationally by definition 2 only is an unnecessary limitation. Both of the definitions, originally formulated with a particular example in mind, should find their natural applications.

C. Solubility

Under idealized conditions the variation of protein solubility with pH may be correlated in a simple way with the mean hydrogen ion ionization $\bar{h}$ in solution (50). The derivation is based upon the following assumptions:

1. The chemical potential of the crystalline protein must be constant, i.e. the composition of the crystals must not change with pH.

2. The dissolved protein must be monodisperse at all pH values under consideration.

3. The salt concentration must be constant and so large that the variations of $\gamma_0, \gamma_1, \ldots, \gamma_\nu$ and $\theta_0, \theta_1, \ldots, \theta_\nu$ with pH and with the total protein concentration are so small that they can be neglected.

Let H_rP be the form of the protein whose concentration m_r is maintained at a constant level in the saturated solution. It follows then from equations (53) and (57) that

$$m_t = \frac{\mathfrak{D} m_r}{\left(\dfrac{\mathfrak{D}_r}{m_t}\right)}$$

$$\begin{aligned}\frac{\partial \log_{10} s}{\partial \mathrm{pH}} &= \left(\frac{\partial \log_{10} m_t}{\partial \mathrm{pH}}\right)_{m_r} = -\left(\frac{\partial \ln m_t}{\partial \ln a_{\mathrm{H}}}\right)_{m_r} \\ &= -\frac{\partial \ln \sum\limits_{q=0}^{q=\nu} (1 + \theta_{\nu-q}) a_{\mathrm{H}}{}^{q-r} \dfrac{\gamma_0}{\gamma_q} \prod\limits_{j=0}^{j=q} K^{-1}{}_{\nu-j+1}}{\partial \ln a_{\mathrm{H}}} \\ &= \overline{-q+r} = -(\nu - r) + \bar{h}. \qquad (66)\end{aligned}$$

In equation (66) the total protein concentration m_t is equal to the protein solubility s. $\nu - r$ and $\bar{h}$ are the mean hydrogen ionizations in the crystal and in the solution. It follows directly from equation (66) that the solubility curve has a minimum when $\nu - r = \bar{h}$. In particular it follows that the solubility of isoionic protein is at a minimum at the isoionic point. The rise of the solubility curve on both sides of the isoionic point follows from integration of (66) after introduction of the functional relationship between $\bar{h}$ and pH given by the titration curve obtained in the same medium. If in a region around the isoionic point, pH_0, the titration curve has a constant slope $b^2 = d\bar{h}/d\mathrm{pH}$ the solubility curve of isoionic protein in that same region is a parabola.

$$\frac{\partial \log_{10} s}{\partial \mathrm{pH}} = \bar{h} - (\nu - r) = b^2(\mathrm{pH} - \mathrm{pH}_0)$$

$$\log \frac{s}{s_0} = \frac{1}{2} b^2(\mathrm{pH} - \mathrm{pH}_0)^2 \tag{67}$$

where s_0 is the solubility at the isoionic point.

It has been pointed out (50) that the molecular weight Mw of the protein may be determined from equation (66). The value of the logarithmic derivative is independent of the units in which s is given whereas $\bar{h}$ and $\nu - r$ are numbers of protons dissociated *per molecule*. Thus

$$\frac{\partial \log_{10} s}{\partial \mathrm{pH}} = \mathrm{Mw}(\bar{h}' - (\nu' - r')) \tag{68}$$

where $\bar{h}'$ and $\nu' - r'$ are numbers of protons dissociated *per one gram* of protein. These values are experimentally obtainable from a titration curve.

Among the three assumptions made in the derivation of equation (66), number one is the most restrictive. In Section VII we shall consider the variation of the solubility of β-lactoglobulin with pH and ionic strength.

VI. Molecular Interpretation of Protein Acid-Base Equilibria

A. Introduction

In the preceding section the protein was considered to exist in a number of forms characterized only by the number of protons and other ions and solutes bound to it. This was sufficient for the purpose of thermodynamic description of the titration curve. The form H_iP contains a total of i dissociable protons per molecule and its concentration m_i comprises all protein molecules with i dissociable protons, irrespective of their positions on the molecule. If each molecule has a total of ν different sites to which protons may be bound, the i protons in H_iP may be distributed among the ν sites in $\nu!/i!(\nu - i)!$ different ways. Many of these are of course very unlikely to occur. Thus a distribution where the main part of the carboxyl and amino groups are both uncharged will be virtually absent in solution. It has been calculated (1) that the distribution of protons just among the histine residues in isoelectric hemoglobin in which 12 of the 33 histidine residues have lost protons corresponds to

$$\frac{33!}{21!\cdot 12!} = 354{,}817{,}320$$

different forms, all of which have a reasonable chance of existence in solution. Generally we have to think of a protein solution as composed at any moment of a great number of "chemically distinct" forms both as regards the number of protons bound to a single molecule as well as the distribution of these protons among the proton-binding sites on the molecule.

In this section we shall look at the protein at the molecular level. We shall consider in detail the interpretation of protein acid-base equilibria in terms of molecular properties. As stated in the introduction, present knowledge of protein structure in solution is entirely inadequate for a rigorous construction of the titration curve from molecular data. Such an undertaking would also meet with other difficulties not especially connected with proteins but rather with solution theory in general. As an illustration of this state of affairs we may point to the semiempirical nature of existing theories of intramolecular charge interactions in aqueous solution discussed in Section IV. The general approach, therefore, has been to adopt a "model" of the protein molecule in solution. In the model all relevant properties (molecular structure, mutual interactions between ionizable groups, etc.) are assumed to be known and a calculation of the titration curve for the model protein solution is possible. Subsequent comparison with experimental results may then furnish valuable information on protein structure and composition. Usually a number of different models can be made to fit the titration curve of a given protein and additional evidence is needed to distinguish between them. In the absence of contradictory evidence from other sources the simplest model which will account satisfactorily for the titration curve is usually considered to be the most successful.

In evaluating the acid-base equilibria from molecular data we shall use the distribution function[7]

$$f_j = \frac{e^{-\epsilon_j/kT}}{\sum_i e^{-\epsilon_i/kT}} \tag{69}$$

f_j is the probability of finding a closed system which is immersed in a thermostat of temperature T (a canonical ensemble) in the state j with energy ϵ_j. The summation in the denominator is over all possible states of the system.

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[7] For a straightforward and concise introduction to the distribution function in statistical thermodynamics, see e.g. E. Schrödinger, "Statistical Thermodynamics," Cambridge Univ. Press, London and New York, 1948.

Let us consider a solution A of a pure protein which is so dilute that interactions between protein molecules, N in number, can be neglected. The thermodynamic behavior of such a solution is amply described by an ensemble of N identical, closed systems B in thermal contact, each containing one protein molecule dissolved in the average amount of solvent per protein molecule in solution A. Using the distribution function [equation (69)] on one of the small systems B we can immediately write down an expression for the ratio of the concentrations of any two forms of the protein, say H_iP and $H_{i+1}P$, which is equal to the ratio of the probabilities of finding the protein molecule in the forms H_iP and $H_{i+1}P$. We get, using also equation (49) (valid in the absence of ion binding)

$$\frac{a_H\gamma_i}{K_{\nu-i}\gamma_{i+1}} = \frac{m_{i+1}}{m_i} = \frac{\sum_j^{\Omega_{i+1}} f_j}{\sum_j^{\Omega_i} f_j} = \frac{\sum_j^{\Omega_{i+1}} e^{-\epsilon_j/kT}}{\sum_j^{\Omega_i} e^{-\epsilon_j/kT}} \tag{70}$$

where the summations are over Ω_i and Ω_{i+1} quantum states compatible with the existence of the forms H_iP and $H_{i+1}P$.

Equations of type (70) may be taken as the starting point for a calculation of acid-base equilibria from molecular data. As discussed above the difficulties in evaluating the sums in equation (70) make it necessary to introduce model solutions. In the construction of suitable models the guiding principles are the available information on protein structure and on intramolecular interactions between ionizable groups. The nature of these interactions has already been discussed in Sections III and IV. Due to their general importance, long-range electrostatic interactions naturally belong to any reasonable protein model, but as pointed out in Section III, additional short-range interactions, e.g. hydrogen bonding, may significantly influence the acid-base equilibria of some protein solutions. The importance of such interactions, however, probably vary strongly from protein to protein, whereas the importance of electrostatic interactions is a general one. It is, therefore, natural to try first a purely electrostatic model for the interpretation of a given titration curve. If no good fit of such a model to the data can be obtained or if the electrostatic model proves to be unreasonably complicated, incorporation into the model of other interactions can be tried. Along these lines Laskowski and Scheraga in a thorough study (28) have estimated the effect of hydrogen bonding between protein side groups. Other modifications may of course also turn out to be profitable.

We shall limit ourselves here to a discussion of purely electrostatic models which are in fact often quite successful in the interpretation of protein titration curves. Some selected examples in Section VII will illustrate this as well as the breakdown of simple electrostatic models in accounting for the titration curve of serum albumin. The presence of salt complicates the situation considerably, and we shall therefore begin by assuming that the ionic strength is vanishingly small (zero). This of course is not always an attainable experimental situation, particularly in acid and alkaline solution, but often an extrapolation to zero ionic strength is possible. The results in the following, derived under the assumption of zero ionic strength, should be compared with such extrapolated experimental values.

B. Rigid Impenetrable Spheres at Zero Ionic Strength

Measurements of the sedimentation and diffusion rate (51), light scattering (52), and hydrogen exchange (53), etc., of protein solutions indicate that many globular proteins can be regarded as fairly rigid structures whose hydrodynamic behavior is similar to that of spheres or slightly elongated ellipsoids. In the first attempt by one of the authors in 1924 to interpret the titration data of globular proteins on the basis of simple assumptions the protein molecule was pictured as a rigid sphere (4). The spherical shape has also been maintained in some later models (39, 45) and probably represents many globular proteins quite well in this context compared with, for example, a somewhat elongated ellipsoid which is much harder to handle mathematically.

More critical than the "smoothed" shape are the assumptions made concerning the positions and the electrostatic interactions of the charged groups in the model. The average dielectric constant D_i in the interior of the protein molecule must be quite low. From percentages of hydration (51, 54) (a figure of 20% is often assumed) we may roughly estimate D_i to be less than one-tenth of that of water and probably lower. The ionization of a group located in the interior of the molecule well below its surface, whereby a proton is lost to, or received from, the surrounding solvent, is therefore exceedingly more difficult than if the same group were located at the surface. Thus it has been customary to assume that all ionizable groups active in the acid-base equilibria are located at or near the surface of the molecule. Ionizable groups that are "buried" in the interior of a rigid protein molecule may become protolytically active, however, if the native folding of the peptide chain is altered due to a change in environment whereby these formerly "buried" groups become exposed to solvent.

The interactions between charged groups in the models under discussion are "electrostatic." This means that the standard free energy [equation (39)] of dissociation of an acid group j in the model, with the charges on all other groups kept constant, is written as the sum of a constant term $\Delta F^\circ_{\text{int}}$ corresponding to the intrinsic dissociation constant K_j^* and an "electrical" term $\Delta F^\circ_{\text{el}}$

$$-kT \ln K = \Delta F^\circ = \Delta F^\circ_{\text{int}} + \Delta F^\circ_{\text{el}} = -kT \ln K_j^* + \Delta F^\circ_{\text{el}} \tag{71}$$

where

$$F^\circ = F^\circ_{\text{int}} + F^\circ_{\text{el}} \tag{72a}$$

$$F^\circ_{\text{int}} = -kT \sum_j^p \ln K_j^* \tag{72b}$$

$$F^\circ_{\text{el}} = \sum \frac{\epsilon_k \epsilon_l}{r_{kl} D_E} \tag{72c}$$

The summation in equation (72b) extends over all groups from which protons have been dissociated, and the summation in equation (72c) extends over all pairs of charges. r_{kl} is a charge-charge distance and D_E is an effective dielectric constant. The success of simple electrostatic theory in accounting for the activity coefficients of dilute solutions of electrolytes (Debye-Hückel theory) and the discussion in Section IV of electrostatic interactions in simple dibasic acids suggests that at sufficiently large distances between charges F°_{el} and D_E can be calculated from simple electrostatics as applied to uniform, continuous media in Section I. As discussed previously the situation is different at small charge-charge distances (of the order of magnitude $r < 10$ Å., compare Table VI). Then semiempirical theories only are presently available for the charge-charge interaction in concentrated electrolyte solutions (55) or for the charge-charge interaction in short-chain dicarboxylic acids, and, as a matter of fact, the simple, purely electrostatic origin of these interactions may be questioned (56). In globular proteins like ovalbumin and β-lactoglobulin the average surface area per ionizable group per molecule is of the order of 100 Å^2. (40). The construction of reasonable electrostatic protein models, even of known charge configuration, therefore meets with great difficulty in the sense that no *a priori* calculations in general are possible. It is in full accord with this that the significance of the experimentally determined values of the parameter ω_0 in equations (80) and (91) below in indicating molecular dimensions is taken with some reservation (57, 58).

Using equations (71) and (72) the general expression (70) may be considerably simplified if the summations are carried out in two steps. In the first step the charges on individual ionizable groups are kept constant, and in the second step the summation is carried further by summing over all distributions of the net charge on the available ionizable groups per molecule. Let us further assume that the protein contains only one type of ionizable group, e.g. all carboxyl groups with the same intrinsic dissociation constant, K^*. The following equations also apply if the protein contains two or more types of ionizable groups, e.g. carboxyl and guanidyl groups, with widely different K^* values, so that the variation in the distribution of charge between the two types of groups taken as a whole can be neglected. One obtains, remembering that the ionic strength is zero,

$$\frac{\sum_j^{\Omega_{i+1}} e^{-\epsilon_j/kT}}{\sum_j^{\Omega_i} e^{-\epsilon_j/kT}} = \frac{\sum^{\rho_{i+1}}\sum e^{-\epsilon_j/kT}}{\sum^{\rho_i}\sum e^{-\epsilon_j/kT}} = \frac{\sum^{\rho_{i+1}} e^{-F^\circ/kT}}{\sum^{\rho_i} e^{-(F^\circ+\mu)_{\mathrm{H}}/kT}} = \frac{a_{\mathrm{H}}}{K^*}\frac{\sum_l^{\rho_{i+1}} e^{-\psi_l/kT}}{\sum_l^{\rho_i} e^{-\psi_l/kT}} \tag{73}$$

where $\psi_l = F^\circ_{el}$ is the electrical free energy at zero ionic strength of the configuration, l, specifying the charge on individual ionizable groups. ρ_{i+1} and ρ_i are the numbers of such configurations compatible with the existence of $H_{i+1}P$ and H_iP, respectively. From equations (70) and (73)

$$K_{\nu-i} = K^* \frac{\sum_l^{\rho_i} e^{-\psi_l/kT}}{\sum_l^{\rho_{i+1}} e^{-\psi_l/kT}} \tag{74}$$

If there is a total of ν ionizable groups per molecule

$$\frac{\rho_i}{\rho_{i+1}} = \frac{\binom{\nu}{i}}{\binom{\nu}{i+1}} = \frac{i+1}{\nu-i} \tag{75}$$

and

$$K_{\nu-i} = \frac{i+1}{\nu-i} K^* e^{-(\psi^{(i)}-\psi^{(i+1)})/kT} \tag{76}$$

where

$$e^{-\psi^{(i)}/kT} = \overline{\left(\sum_l^{\rho_i} e^{-\psi_l/kT}\right)} \tag{77}$$

(the bar denotes the average over all ρ_i configurations). $\psi^{(i)}$ and $\psi^{(i+1)}$ are thus the average molecular electrical free energies of the forms H_iP and $H_{i+1}P$ at zero ionic strength. Therefore, if $\psi^{(i)} - \psi^{(i+1)}$ can be determined, substitution of equation (76) in equation (54) yields $\bar{h}$ as a function of a_{H}, i.e. the titration curve.

To calculate $\psi^{(i)} - \psi^{(i+1)}$ one of the authors suggested a model (4) [see also (58)] that treated the protein molecule as a rigid sphere charged evenly on the surface with the net charge of the molecule and immersed in solvent having a dielectric constant equal to the bulk dielectric constant of water D_e. The continuous and uniform charge distribution in this "smeared site" model is equivalent to the assumption that all ionizable groups are randomly distributed on the surface, i.e. they occupy any position on the surface with equal probability. At zero ionic strength the electrical energy of such a sphere of radius b and charge $s\epsilon$ (ϵ = electronic charge) is

$$\psi_0 = \frac{s^2\epsilon^2}{2bD_e} \tag{78}$$

Putting

$$s = h = \nu - i \tag{79}$$

for the molecule under consideration containing only carboxyl groups,

$$\begin{aligned} K_{\nu-i} &= \frac{i+1}{\nu - i} K^* e^{-\omega_0[(\nu-i)^2-(\nu-i-1)^2]} \\ &= \frac{i+1}{\nu - i} K^* e^{\omega_0(1-2\nu-2i)} = \frac{i+1}{\nu - i} K^* e^{\omega_0(1-2h)} \end{aligned} \tag{80}$$

where

$$\omega_0 = \frac{\epsilon^2}{2kTD_e b} \tag{81}$$

An interesting discussion of equation (80) is given by Gurd and Wilcox (44). Substitution of equation (80) into equation (54) completes the construction of the titration curve for this model at zero ionic strength.

The resulting expression is quite complicated and impractical for comparison with experimental data. It is therefore very important that simpler, approximate expressions have been found. It was pointed out (4) that near the mid-point of the titration curve the slope is

$$\frac{d\bar{h}}{d\text{pH}} = \frac{2.303\nu}{2(\omega_0\nu + 2)} \tag{82}$$

Cannan (59) has shown by numerical calculations that the equation

$$\text{pH} = \text{p}K^* + \log\frac{\bar{h}}{\nu - \bar{h}} + 0.868\omega_0\bar{h} \tag{83}$$

is a very good approximation to equation (54), substituted with (80), over most of the titration curve. The approximation improves with increasing number of ionizable groups. In typical examples Cannan found it to be better than 0.1 equivalent per mole of protein over the whole of the titration curve. Differentiation of equation (83) at the mid-point, $\bar{h} = \nu/2$, gives equation (82). Titration equations of which (83) is a special case have been derived theoretically by Overbeek (60) and Katchalsky and Gillis (61) (see below).

A complication arises when the macromolecule contains more than one type of ionizable group, especially when the pH zones of ionization of different types overlap, as, for example, for imidazole and α-amino groups. This situation has been treated by Kirkwood (45) and Putzeys and Bouckaert (62), using equation (78) to calculate the electrical free energy; and by Hill (63), and Tanford and Kirkwood (39, 40), using more general expressions for the electrical free energy to be discussed below. It can, however, be shown that the titration curve of some electrostatic protein models, which contain several types of groups, in dilute solution is given approximately by the superposition of the titration curves for the individual types of groups given by expressions similar to equation (83) [cf. reference (64)].

To prove this we make the assumption that all charged groups are at the same average electrostatic potential which is proportional to the net charge $z° - h$ of the protein molecule. The proportionality factor may, however, vary slowly with the net charge. Consider then one ionizable group g on the protein with intrinsic acid dissociation constant $K_g{}^*$. This group can exist in two forms, a protonated acid form and a basic form. Let α_g be its degree of proton dissociation. Expressing $(1 - \alpha_g)/\alpha_g$ in terms of the distribution function (equation 69) we find in analogy with equation (73)

$$\frac{1 - \alpha_g}{\alpha_g} = \frac{\sum^{\theta_g}\sum^{A} e^{-\epsilon_i/kT}}{\sum^{\theta_g}\sum^{B} e^{-\epsilon_i/kT}} = \frac{\sum_r^{\theta_g} e^{-F_r/kT}}{\sum_r^{\theta_g} e^{-F_r'/kT}} \tag{84}$$

where F_r and F_r' are the free energies of the system when the group g is in its acid and base form, respectively, and the charge distribution r among the remaining groups is specified. The summations are over all θ_g possible distributions r. F for the system containing one protein molecule can be written

$$F = F° + hkT \ln a_{\mathrm{H}} \tag{85}$$

Taking the logarithm on both sides of equation (84) and using the approximation

$$\ln \sum_r^{\theta_g} e^{-F_r/kT} - \ln \sum_r^{\theta_g} e^{-F_r'/kT} \sim -\frac{\partial \ln \sum_r^{\theta_g} e^{-F_r/kT}}{\partial h}$$

$$= \frac{1}{kT} \frac{\sum_r^{\theta_g} \partial F_r/\partial h \, e^{-F_r/kT}}{\sum_r^{\theta_g} e^{-F_r/kT}} = \frac{1}{kT} \overline{\left(\frac{\partial F}{\partial h}\right)}$$

we obtain from equations (72) and (85)

$$\ln \frac{1 - \alpha_g}{\alpha_g} = \frac{1}{kT} \overline{\left(\frac{\partial F}{\partial h}\right)} = -\ln K_g^* + \ln a_{\mathrm{H}} + \frac{1}{kT} \overline{\left(\frac{\partial F^\circ_{\mathrm{el}}}{\partial h}\right)} \tag{86}$$

which after rearrangement and multiplication with 0.434 gives

$$\mathrm{pH} \simeq -\log a_{\mathrm{H}} = \mathrm{p}K_g^* + \log \frac{\alpha_g}{1 - \alpha_g} + \frac{0.434}{kT} \overline{\left(\frac{\partial F^\circ_{\mathrm{el}}}{\partial h}\right)} \tag{87}$$

The average in equation (87) is over all charge distributions among ionizable groups in the molecule. If the number of ionizable groups per molecule is large, equation (63) shows that we need to average only over charge distributions for which $h/\bar{h} \sim 1$. According to assumptions

$$\frac{\partial F^\circ_{\mathrm{el}}}{\partial h} = \beta(z^0 - h);\ \beta = \text{constant};\ \frac{h}{\bar{h}} \sim 1 \tag{88}$$

so that

$$\overline{\left(\frac{\partial F^\circ_{\mathrm{el}}}{\partial h}\right)} = \beta(z^\circ - \bar{h}) = \frac{\partial(\overline{F^\circ_{\mathrm{el}}})}{\partial \bar{h}} \tag{89}$$

which after substitution into equation (87) gives

$$\mathrm{pH} = \mathrm{p}K_g^* + \log \frac{\alpha_g}{1 - \alpha_g} + \frac{0.434\beta}{kT}(z^\circ - \bar{h}) \tag{90}$$

equation (90) is the approximate equation for the titration curve of group g. It is also the equation for the titration curve of all groups of a given type, characterized by the intrinsic dissociation constant K_g^*, the fraction of dissociated groups of this type being α_g. Finally, the complete titration curve of this protein model is obtained as the superposition of as many curves of type equation (90) as there are different types of groups. If the expressions (equation 78) for $F^\circ_{\mathrm{el}} = \psi_0$ of the "smeared site" model is introduced in equation (87), equation (90) becomes

$$\mathrm{pH} = \mathrm{p}K^* + \log \frac{\alpha}{1 - \alpha} - 0.868\omega_0(z^\circ - \bar{h}) \tag{91}$$

equations (90) and (91) thus have the same form, except for the last, electrical term, as the ordinary equation for the titration curve of a monovalent acid. It is interesting that Hartley and Roe (65) have pointed out that the surface potential [equation (89)] of the "smeared site" model may be obtained independently from electrophoresis studies as the so called electrophoretic potential (see Chapter I of this book).

Equations (90) and (91) are so simple that one can fairly easily adjust their parameters (β or ω_0, the number of different types of ionizable groups, their intrinsic dissociation constants, and the number of groups belonging to each type) to make a best fit to a given titration curve. Equation (91) has been used frequently in the past in protein titration work. In several cases (Section VII) it accounts very well for the whole or parts of the titration curve with parameter values that are in reasonable agreement with what is known about the protein from other sources.

This success of equation (91) could not have been expected because of the unrealistic assumptions on which it is based. To derive (91) we had to assume that all ionizable groups are randomly distributed on the surface of a rigid sphere ("smeared site" model) and that their mutual interaction can be calculated using the bulk dielectric constant of water. Both assumptions are incorrect. The ionizable groups of a globular protein are mostly located on side chains extending into the solvent from a structure of tightly coiled peptide chains. The charges of the protein molecule are, therefore, confined to relatively small regions on the molecular surface, the extent of which is determined by the flexibility of the side chains, and they can not be considered as randomly distributed on the surface. In the calculation of the mutual interactions between charged groups in a hypothetical spherical protein molecule with groups randomly distributed on the surface, the use of the bulk dielectric constant of water implies that the interactions are indeed purely electrostatic and that electrical saturation effects, etc., can be neglected. Thus we may conclude that the "smeared site" model in a fortunate way combines two crude assumptions, the first concerned with distribution of charged groups and the second, with the magnitude of their mutual interactions. Apparently, in the number of proteins for which equation (91) successfully accounts for the whole or parts of the titration curve, the assumption of randomly distributed charges overestimates the real charge-charge interaction to approximately the same degree as the use of the bulk dielectric constant of water underestimates it, the final result being approximately correct.

Tanford and Kirkwood (39, 40) have recently tried to put the interpretation of the acid-base equilibria of globular proteins on firmer ground by proposing a more realistic model than the "smeared site" model. Their model is based on Kirkwood's (11) earlier cavity model. It has already been presented in Section I and discussed in detail in Sections I and IV. The protein molecule is treated as a sphere with dielectric constant 2.0 in which point charges corresponding to the charged groups are located at fixed positions near the surface. The intramolecular interactions between ionizable groups follow from purely electrostatic calculations of the electrical energy of the model, i.e. F°_{el} of equations (71) and (72) at zero ionic strength is given by ψ_0 of equation (16). For a model with ionizable groups of only one type with intrinsic dissociation constant K^*, substitution of the calculated electrical free energies F°_{el} in equation (74) gives the acid dissociation constants of the model as a whole from which the titration curve may be calculated using equation (54). To calculate the sums (partition functions) in equation (74), Tanford and Kirkwood use an approximation and they also treat the general case of several types of ionizable groups per molecule. For details we refer to the original paper (39).

The application of the general equations obtained (39) to simple model molecules, constructed to resemble actual proteins, yields some interesting results (40). Plotting pH $-\log \alpha/(1-\alpha)$ against $\bar{z}$, where α is the degree of dissociation of a given type of group, gives almost linear plots, the slope ω_0' of which is markedly dependent on the depth d of the charges below the surface (Section IV). For eight carboxyl groups distributed uniformly on the surface of a sphere of radius 10 Å., $\omega_0' \approx 0.5\omega_0$ where ω_0 is the corresponding slope given by equation (81) of the same type of plot [equation (83)], using a "smeared site" model. Taking ω_0 as the experimentally correct value, it was found that the charges had to be placed approximately 1 Å. below the surface of the sphere in order to make $\omega_0' = \omega_0$. The value of $d \simeq 1$ Å. is also found experimentally for the cavity models of many dicarboxylic acids (Table VI), and it might, therefore, for a moment be considered as a kind of constant associated with the carboxyl group (37, 40). However, the value of d required to make $\omega_0' = \omega_0$ varies with the group density on the surface, and it would appear that more data are necessary before $d = 1$ Å. can be considered as a constant associated with the carboxyl group in the cavity model. We refer to the discussion of electrostatic interactions presented in Section IV.

The main advantage of a protein model with discrete charges is that the effect of varying relative charge positions can be investigated and the result compared with equation (78) or (88), according to which the electrical energy is proportional to the square of the net charge. Tanford (40) has shown by numerical calculations on selected models that if all ionizable groups in the cavity model are placed at a constant depth of 1 Å. below the surface, considerable variation in calculated titration curves may still arise as a result of the distribution of ionizable groups with respect to one another. He made the important conclusion that many of the "anomalies" of experimental titration curves may be accounted for on the basis of electrostatic interactions alone. Thus a "crowded" distribution of carboxyl groups would lead to an increased ω_0' compared with that of a uniform distribution of the groups. In basic proteins ω_0' may be largely determined by the interactions between the negatively charged carboxylate groups and the positively charged amino and guanidyl groups. If the distribution of groups is such that the interaction with positive groups varies from one carboxylate group to the next, an increase in ω_0' is also the result. Similarly, the intercept of a pH $-\log \alpha/(1-\alpha)$ plot with the ordinate axis $\bar{z} = 0$ may vary with the distribution of ionizable groups in the model. In the "smeared site" model the intercept corresponds to the intrinsic dissociation constant K^*, but this is not necessarily true in a model with discrete charges where the electrical energy does not vanish with vanishing net charge. In this way the small variations in apparent intrinsic dissociation constants, e.g. for carboxyl groups, determined from equation (91) (see Table IV) finds a simple explanation. Of course this phenomenon might also be given a nonelectrostatic explanation along the general lines of Section III. The influence of the distribution of ionizable groups on the titration curve has been discussed earlier by Hill (66), who also discussed a spherical protein model with discrete charges located in some regular array in which only interactions between nearest neighbors are counted (63).

C. Rigid Impenetrable Spheres at Finite Ionic Strength

The effect of counterions and added salt on the titration curve may theoretically be calculated from equation (54) in terms of the activity coefficients $\gamma_0, \gamma_1, \ldots, \gamma_\nu$ and the ion binding parameters $\theta_0, \theta_1, \ldots, \theta_\nu$ when the thermodynamic dissociation constants $K_1, K_2, \ldots, K_\nu$ are known. Neglecting ion binding for a moment the activity coefficients γ for a spherical cavity model with arbitrary charge distribution follow from equation (17) valid at low ionic strengths (10, 11). For the "smeared site" model, equation (17), reduces to equation (19) which, together with equations (14) and (78), yields the electrical free energy ψ of the "smeared site" model at low ionic strengths (4)

$$\psi = \psi_0 + kT \ln \gamma = \frac{s^2\epsilon^2}{2bD_e} - \frac{s^2\epsilon^2}{2D_e}\frac{\varkappa}{1 + \varkappa a} = s^2kT\omega \tag{92}$$

where

$$\omega = \frac{\epsilon^2}{2D_ekT}\left[\frac{1}{b} - \frac{\varkappa}{1 + \varkappa a}\right] \tag{93}$$

Substitution of equation (92) in (76) and (87) gives, as above, expressions for the acid dissociation constants and for the ionization of a given type of group of the same forms as equations (80) and (91), but with ω substituted for ω_0. The general shape of the titration curve of the "smeared site" model, therefore, is the same as at zero ionic strength, but owing to shielding of charges by small ions the slope $d\bar{h}/d\text{pH}$ is steeper

The characteristics of the cavity model with discrete charges is also preserved at low ionic strengths where equation (19) containing the net charge of the molecule is still a good approximation for the activity coefficient (40). At higher ionic strengths the complete equation (17) must be used.[8] However, as the ionic strength increases equations (17) and (19 will become less and less applicable as the linearized Poisson-Boltzmann equation (1) fails to describe the potential distribution. The complete Poisson-Boltzmann equation can be integrated numerically [compare reference (74)]. How much of an improvement such a solution constitutes over equations (17) and (19) is, however, not obvious as the Poisson-Boltzmann equation itself is open to criticism (6–8). Experimentally it is found that titration data at various ionic strengths up to 0.1 or higher can be interpreted using equation (93) with reasonable values for the radius of the protein molecule b and the radius of the small-ion exclusion volume a (see Section VII).

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[8] It should be noted that it follows from equation (17) that the deeper the charges are located in the cavity, the less effective is added salt in shielding the charges in the cavity.

Many proteins bind small ions especially when their net charge z has a large numerical value. Ion binding may be inferred from equilibrium dialysis experiments (75), electromotive force measurements (76, 77), electrophoretic mobility studies (78, 2), titrations with various acids (79), or from pH changes following on addition of neutral salt (48). We shall not attempt a discussion of these methods of measuring ion binding or their interpretation but refer to a recent detailed account by Edsall and Wyman (80). The effect on protein acid-base equilibria of ion binding in the "smeared site" model is simply to change the net charge of the molecule by an amount equal to the total net charge of the bound ions. For such a model to be applicable the ion binding "sites" must not cluster on the surface of the protein molecule and short range interactions between the type of groups, e.g. COOH, under consideration and the bound ions must be weak. In particular, competition between hydrogen ions and metal ions for a binding site must be absent. For this "smeared site" model with ion binding at low ionic strength, equation (91), assumes the form (compare equation (64))

$$\begin{aligned}\mathrm{pH} &= \mathrm{p}K^* + \log\frac{\alpha}{1-\alpha} - 0.868\omega(z^\circ - \bar{h} + y)\\ &= \mathrm{p}K^* + \log\frac{\alpha}{1-\alpha} - 0.868\omega\bar{z} \qquad (94)\end{aligned}$$

where $z^\circ - \bar{h} + y$ is the net charge z of the protein and y is the charge contribution from bound ions other than H^+. Just as equation (91) accounts for many protein acid-base equilibria at zero ionic strength, equation (94) accounts for these same equilibria in many cases of ion binding. Thus Scatchard and co-workers (48, 81) have shown that the displacement of the isoionic point $z^\circ - \bar{h} = 0$ of serum albumin on addition of NaCl, NaI, NaSCN, etc. is in fair agreement with data for the binding of Cl^-, I^-, SCN^-, etc., obtained independently by one or more of the other methods listed above. The change in isoionic point of a 1% serum albumin solution on salt addition follows directly from equation (94) if it is noted that α and $\bar{h}$ vary but little in the process (48).

$$\Delta\text{pH} = -0.868\omega(\Delta\bar{z}) = -0.868\omega\Delta y \tag{95}$$

D. Other Models

Calculations based on various electrostatic polyelectrolyte models have been published. Fuoss and associates (82) have solved the Poisson-Boltzmann equation for the electrical potential of infinitely long, cylindrical, and ionized polyelectrolyte molecules in solution without added salt, [see also (83)]. The case of added salt has been treated by Hill (84). Calculations are also available for rigid, impenetrable, and ellipsoidal models (10, 85). Finally we shall mention the synthetic polyelectrolytes which have been the subject of intensive study during the last decade. Their behavior is in general more complicated than that of globular proteins. Thus a theoretical treatment of the acid-base equilibria of, for example, polyacrylic acid has to take into account also the flexibility of this molecule, whereas many globular proteins can be considered as fairly rigid structures that change but little with the degree of ionization. Several flexible polyelectrolyte models have been constructed. Although more complex than the rigid protein models discussed above, the "electrostatic" part of the theory is common to both models and the results obtained with synthetic polyelectrolytes may therefore eventually be used on proteins. The early work is reviewed by Strauss and Fuoss (86), and Schneider and Doty (87) have critically examined some polyelectrolyte models in the light of experimental data obtained with solutions of sodium carboxymethyl cellulose. This study led Harris and Rice (41, 88) to the formulation of new models. We refer to these papers for the many references to earlier theories.

VII. EXPERIMENTAL TITRATION CURVES

As an illustration of the application of the models presented we shall discuss in this section the titration curves of four carefully examined globular proteins: ovalbumin, β-lactoglobulin, ribonuclease, and serum albumin. The emphasis will be on how well a model accounts for the experimental titration curve [in all four cases listed equation (94) was used]. We shall not be concerned with experimental techniques nor with the trial-and-error method of fitting a particular model to the experimental titration curve. For details of procedure we refer to references (1-3, 57, 80). It should, however, be pointed out that particular attention must be paid to an examination of whether a given titration curve is reversible or not, i.e. if the back titration curve coincides with the original titration curve. A deviation between the two indicates that part of what is being measured corresponds to irreversible changes which then call for special attention.

The discussion in Sections V and VI dealt only with true equilibria, i.e. reversible titration curves. The requirement of reversibility usually limits the pH range over which titration curves can be obtained. Thus reversible titration curves for ovalbumin were obtained from pH 2 to 11.5 (89), but if the pH was increased to about 12.5 rapid irreversible changes took place (90). The fitting of a small number of equations of the form (94) to a given titration curve is usually aided by employing knowledge of protein amino acid composition and by utilizing titration results obtained at various temperatures and in various solvents containing formaldehyde, urea, alcohol, iodine, etc. (1–3).

A. Ovalbumin

Cannan and associates (91) have studied the titration of ovalbumin in much detail at various temperatures and ionic strengths. The effect of addition of KCl is to increase the slope of the titration curve so that the numerical value of the corresponding change in pH, ΔpH_s, at constant $\bar{h}$ vanishes near the isoelectric point as reported earlier by Sørensen (47, 92). They found that if the titration curve in 0.067 M KCl is taken as the standard of reference, ΔpH_s is a linear function of the net charge $\bar{z}$ of the molecule ($\bar{z}$ is calculated without correction for ion binding)

$$\Delta pH_s = \alpha_s \bar{z} + \beta_s \tag{96}$$

α_s and β_s are constants for a given KCl concentration. $\beta_s \neq 0$ is the expression for the fact that the titration curves at different KCl concentrations do not intersect at the isoionic point 4.9 in 0.067 M KCl. Encouraged by the simple relation, equation (96), Cannan *et al.* displaced their titration curves along the pH axis so that they all intersected at pH 4.9. This new system of titration curves was then interpreted successfully using equation (91). Before discussing the results of their analysis it may be pointed out that equation (96) follows directly from equation (94) if β_s is the result of a pH-independent ion binding.

The ionization curves (pH 2–11) are consistent with the presence of 51 carboxyl, 5 imidazole, 23 amino, and 14 guanidyl groups in a molecule of molecular weight 45,000. The whole of the experimental titration curve can be reproduced, apparently within experimental error, by the superposition of three curves of the form equation (91), all with the same value of $\omega = \omega_{exp}$. The first curve describes the ionization of 51 groups with $pK^* = 4.29$; the second the ionization of 5 groups with $pK^* = 6.7–6.8$; and the third curve describes the ionization of 23 groups with $pK^* = 10.07$. In Table VII the value of ω_{exp} is compared with corresponding values of ω calculated on the basis of the "smeared site" model from equation (93). The radius b of the molecule was taken as 27.5 Å. (the value corresponding to the partial specific volume of ovalbumin and a molecular weight of 45,000). In column ω_1, $a = b = 27.5$ Å. and in column ω_2, $a = 29.5$ Å. and $b = 27.5$ Å.

TABLE VII

COMPARISON OF TITRATION PARAMETERS FOR OVALBUMIN (89)

$\Gamma/2$	ω_{exp}	ω_1	ω_2	ω_{exp}/ω_2
0.0085	0.0585	0.0696	0.0717	0.82
0.0167	0.0505	0.0592	0.0621	0.81
0.033	0.0425	0.0484	0.0523	0.81
0.067	0.0345	0.0385	0.0434	0.80
0.133	0.0285	0.0299	0.0358	0.80
0.267	0.024	0.0227	0.0294	0.81
0.667	0.019	0.0153	0.0230	0.83
1.19	0.0185	0.0118	0.0200	0.93
2.38	0.0175	0.0086	0.0172	1.0

The ratio ω_{exp}/ω_2 is seen to be constant for $\Gamma/2 < 0.67$. The form of equation (93) is thus adequate for the description of the effect of ionic strength on the titration curve of ovalbumin although the calculated value is too high. As discussed in Section VI it is difficult at present to attach any real meaning to the deviation of ω_{exp}/ω_2 from 1. Recent amino acid analyses of ovalbumin (93) check fairly well with the results derived from the titration curve. However, this agreement may be more or less fortuitous, as ovalbumin is a conjugated protein containing carbohydrate and 1 or 2 molecules of phosphoric acid per molecule of protein. It has no detectable α-amino group and 9 hidden tyrosine groups not reversibly titratable below pH 12 (90). In addition there is evidence that ovalbumin also contains hidden basic groups (94).

B. β-Lactoglobulin

Cannan *et al.* (91) have made a titration study of β-lactoglobulin similar to that carried out on ovalbumin. In their work they assumed a molecular weight of 40,000. More recent values are close to 36,000 (95). Their results are consistent with the presence of 58 carboxyl, 34 amino, 6 imidazole, and 6 guanidyl groups per molecule of molecular weight 40,000. The acid and neutral parts of the titration curve could be accounted for by the superposition of two curves of the form equation (91) with the same value of $\omega = \omega_{exp}$. The first curve describes the ionization of 58 groups with $pK^* = 4.60$ and the second curve the ionization of 6 groups with $pK^* = 6.7–6.8$ Unlike ovalbumin, the alkaline parts of the titration curve of β-lactoglobulin, at various ionic strengths, proved difficult to interpret in terms of the "smeared site" model. The ω_{exp} value in this region turned out to be lower than for the acid and neutral parts of the curve, and no definite value of pK^* for the amino groups could be obtained. Tanford (57) has replotted the data obtained by Cannan *et al.* in a pH $-\log \alpha/(1-\alpha)$ vs. $\bar{z}$ plot where the net charge $\bar{z}$ of the molecule was estimated with the aid of chloride-binding studies by Carr (77).

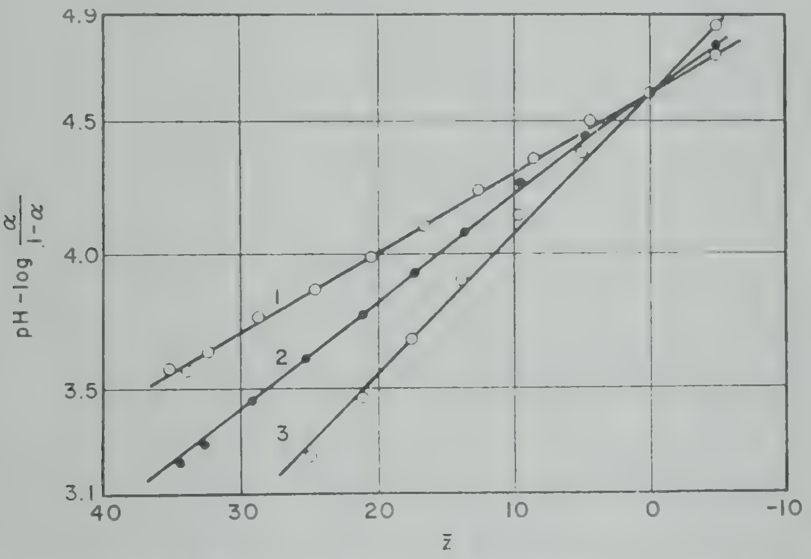

FIG. 4. Titration of the carboxyl groups of β-lactoglobulin. Curve *1*, $\Gamma/2 = 0.270$; curve *2*, $\Gamma/2 = 0.069$; curve *3*, $\Gamma/2 = 0.019$. From Tanford (57).

The points at three different ionic strengths all fall close to a straight line and all three lines intersect at one point at $(\bar{z}, \text{pH}) = (0, 4.60)$ (Fig. 4) as they should when equation (94) is valid.

Cannan *et al.* compared ω_{exp} obtained from the acid branch of the titration curve with ω_{calc} calculated from equation (93) putting $b = 29.1$ Å. and $a = 31.1$ Å. The agreement is surprisingly good for all ionic strengths tried up to 2.1. The deviation nowhere exceeds 6%. The equivalent radius $b = 29.1$ Å. of the molecule is somewhat larger than the corresponding value 23 Å. obtained from the molecular weight 40,000 and the partial specific volume of β-lactoglobulin. If the latter value is used, as for ovalbumin, the ratio $\omega_{exp}/\omega_{calc}$ reduces to approximately 0.8.

The agreement between the numbers of ionizable groups in β-lactoglobulin derived from titration and amino acid analysis is excellent (Table VIII). The titration data of Cannan *et al.* do not permit any conclusions concerning tyrosine ionization.

In Section V, C, it was shown that the solubility of an isoionic protein

TABLE VIII

IONIZABLE GROUPS IN β-LACTOGLOBULIN[a]

	Titration data	Analytical data
Carboxyl	58	58[b]
ϵ – amino	34	32
α – amino + imidazole	6	3 + 3
Guanidyl	6	6

[a] Assumed molecular weight 40,000.

[b] The number of α-carboxyl groups is assumed to be the same as the number of free α-amino groups.

Titration data from Cannan *et al.* (91); analytical data from Tristram (93), recalculated to molecular weight 40,000. The table is based on the table on page 527 of reference (80).

plotted against pH near the isoionic point is a parabola, provided certain assumptions, listed in Section V, C, are made. In particular it was assumed that the composition of the protein crystals did not change with pH. It is clear from the solubility work of Grönwall (96) (Fig. 5) that the equations of Section V, C, are not strictly applicable to β-lactoglobulin. Thus some of the solubility curves of Fig. 5 deviate appreciably from the theoretical curves given by equation (67). Furthermore, the pH of minimum solubility varies with the NaCl concentration, although the isoionic point 5.18 of β-lactoglobulin in solutions of KCl is independent of the salt concentration (91). Binding of Na^+ and Cl^- to β-lactoglobulin is negligible at the isoionic point (77). It was suggested (50) that the data of Fig. 5 might be explained by assuming that the precipitate in the experiments of Grönwall was not isoionic β-lactoglobulin but a salt of β-lactoglobulin. The work of Low and associates (97) on the composition of β-lactoglobulin crystals in various media supports such an explanation.

Although the spherical "smeared site" model accounts well for the acid and neutral parts of the titration curve, it is unable to account for the increase in solubility of isoionic β-lactoglobulin with increasing NaCl concentration (Fig. 5). According to equation (19), applicable to the spherical "smeared site" model, the activity coefficient of a molecule with net charge zero has the activity coefficient 1 independent of the NaCl concentration. The solubilizing action of NaCl on isoionic protein at the isoionic point must therefore be interpreted as an increase in the concentrations of protein molecules carrying net charges different from zero. It can easily be shown (98) that the increase in solubility s of isoionic β-lactoglobulin with increasing NaCl concentration, assuming a "smeared site" model,

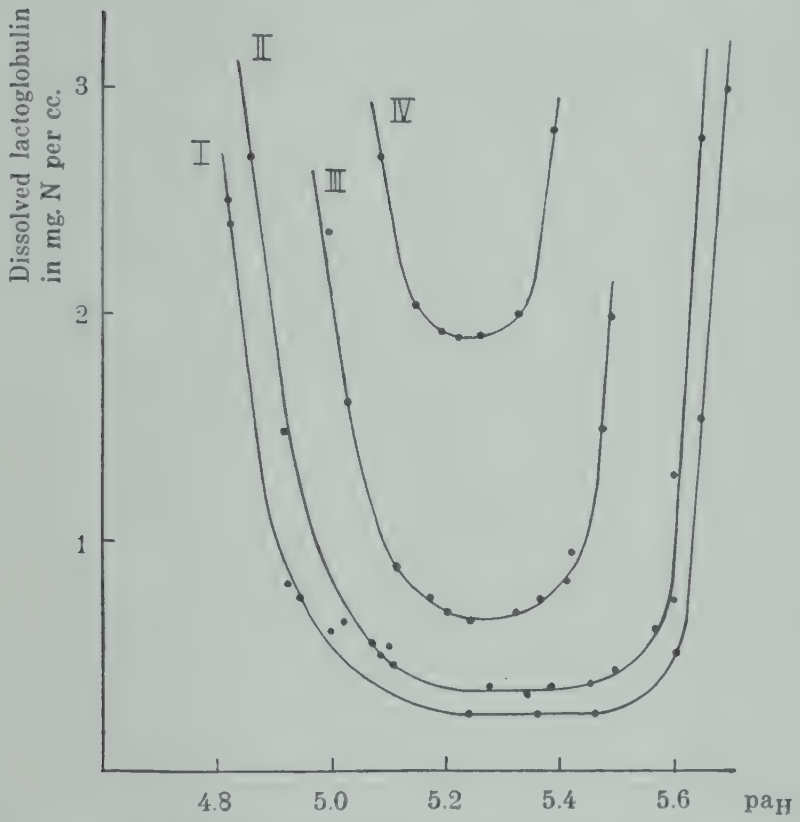

FIG. 5. Solubility of β-lactoglobulin in solutions of NaCl at 25°C. Curve *I*, 0.001 *M* NaCl; curve *II*, 0.005 *M* NaCl; curve *III*, 0.01 *M* NaCl; curve *IV*, 0.02 *M* NaCl. From Grönwall (96).

is given by

$$\left(\frac{\partial \ln s}{\partial \varkappa}\right)_{\mathrm{pH}} = -\overline{\left(\frac{d \ln \gamma}{d\varkappa}\right)} = \overline{(z^\circ - h)^2}\,\frac{\epsilon^2}{2D_e kT}\,\frac{d\left(\dfrac{\varkappa}{1 + a\varkappa}\right)}{d\varkappa}$$

$$= \frac{0.434\epsilon^2}{2D_e kT}\left(\frac{\partial \bar{h}}{\partial \mathrm{pH}}\right)_{\varkappa}\frac{d\left(\dfrac{\varkappa}{1 + a\varkappa}\right)}{d\varkappa} \qquad (97)$$

where $\varkappa$ is the Debye-Hückel parameter given by equation (2). The increase in solubility predicted from equation (97) is an order of magnitude smaller than that actually observed (Fig. 5). The strong dependence of β-lactoglobulin solubility on ionic strength finds a plausible explanation in the electrostatic interaction between the dipole of isoionic β-lactoglobulin and the ion atmosphere (99).

The reason why the titration of the amino groups cannot be accounted for along the same lines as used for the titration of the carboxyl groups is not known. Recent studies (100) of the dissociation of the β-lactoglobulin molecule into two particles, each of approximate molecular weight 17,500 may eventually clarify this point.

C. Ribonuclease

Spectrophotometric (101, 102) and potentiometric (103) titrations of crystalline ribonuclease are in perfect agreement with its completely known amino acid composition (104, 105). Reversible titration experiments can be conducted from pH 2 to about pH 11.5. In this pH range the protein shows no sign of aggregation and it is believed not to bind chloride ions (103). The interpretation of the spectrophotometric titration curves (102), giving information on the ionization of tyrosine phenolic groups, and the interpretation of the potentiometric titration curves pH 2–11.5 are based upon the "smeared site" model [equations (93) and (94)]. It is found that the whole of the titration curve can be accounted for, less a small deviation between pH 9.6 and 10.0, as the superposition of eight curves of the general form equation (91), each with the same value of $\omega = \omega_{\text{exp}}$ and each representing the ionization of a class of groups in the protein molecule. The number of groups in each class and their pK^* values are as follows (103): 1 group (α-COOH), p$K^* = 3.75$; 5 groups (β, γ-COOH), p$K^* = 4.0$; 5 groups (β, γ-COOH), p$K^* = 4.7$; 4 groups (imidazole), p$K^* = 6.5$; 1 group (α-NH_2), p$K^* = 7.8$; 10 groups (ε-NH_2), p$K^* = 10.2$; 3 groups (phenolic), p$K^* = 9.95$; and 4 groups (guanidyl), p$K^* > 12$. Comparisons at the ionic strengths 0.01, 0.03, and 0.15 between ω_{exp} and ω_{calc} (calculated from equation (93) with $a = 19.6$ Å. and $b = 17.1$ Å., which is the equivalent radius of a 20% hydrated molecule with mol. wt. = 13,683 and partial specific volume 0.71) show that the ratio $\omega_{\text{exp}}/\omega_{\text{calc}}$ is equal to 0.80 within a few per cent at all three ionic strengths.

While most of the pK^* values listed are "normal" (see Table IV) the division of the β, γ-carboxyl groups into two classes with pK^* 4.0 and 4.7, respectively, deserves further comment. A $\text{pH} - \log \alpha/(1 - \alpha)$ vs. z plot of the dissociation of *all* carboxyl groups is approximately linear, but with a slope larger than the ω_{exp} referred to above for the ionization of the rest of the groups. A similar situation is encountered in another basic protein lysozyme, but to a higher degree (106). (Isoionic point of lysozyme 11.1; the isoionic point of ribonuclease is at pH 9.6.) It was pointed out by Tanford (40, 103) that the location of charged amino groups in the near neighborhood of some of the carboxyl groups in the basic protein would lead to an increased experimental value of ω compared with that calculated from equation (93). In terms of the "smeared site" model with ω already fixed by other parts of the titration curve, such an experimental situation is describable as an apparent change in pK^* for some of the carboxyl groups (see Section VI, B). The example serves to emphasize the degree of ambiguity that exists in the interpretation of protein titration curves.

A comparison between the amino acid analysis and the titration data shows that all ionizable groups, except 3 tyrosine groups, are reversibly titratable between pH 2 and 11.5. The remaining "buried" tyrosine groups are irreversibly titratable above pH 12 (101, 102), like the tyrosine groups of ovalbumin.

D. Serum Albumin

While many properties of the acid-base equilibria of ovalbumin, β-lactoglobulin, and ribonuclease can be interpreted using equation (94) of the "smeared site" model, the situation in serum albumin is considerably more complex. The physical-chemical properties of serum albumin including its hydrogen ion titration behavior has been—and still is—the subject of intensive investigation, and many important discoveries have been made. However, the interpretation of the highly interesting "anom-

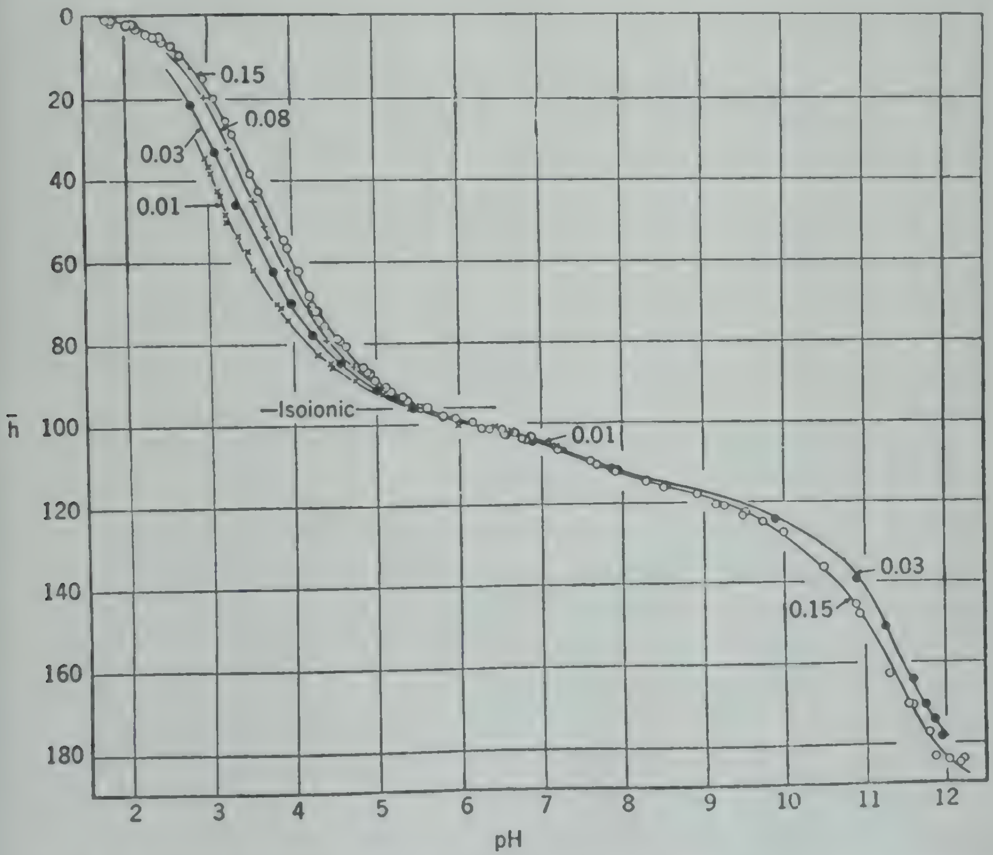

FIG. 6. Titration curves of bovine serum albumin at 25°C. at four ionic strengths. The curves drawn are calculated, using equation (94) and the values of ω given in Fig. 7, together with the pK^* values of Table IV. From Tanford *et al.* (108).

alies" of serum albumin, including its titration behavior in acid solution, is still far from clear. We shall not attempt to review the voluminous, and in part controversial, literature on serum albumin. The acid-base equilibria of serum albumin are discussed in the leading references listed in the beginning of this section (1–3, 57, 80), and Edsall (107) has most recently discussed the physical-chemical properties of serum albumin. We shall merely point out here that attempts (108) to interpret the titration curves of bovine serum albumin (Fig. 6) using equation (94) fail. The theoretical analysis gives good agreement with amino acid analysis and pK values (Table IV) which are on the edge of "normal" values found in other proteins. Furthermore, the thermodynamic functions $\Delta H°$ and $\Delta S°$ for the ionization of phenolic groups are found to be anomalous.

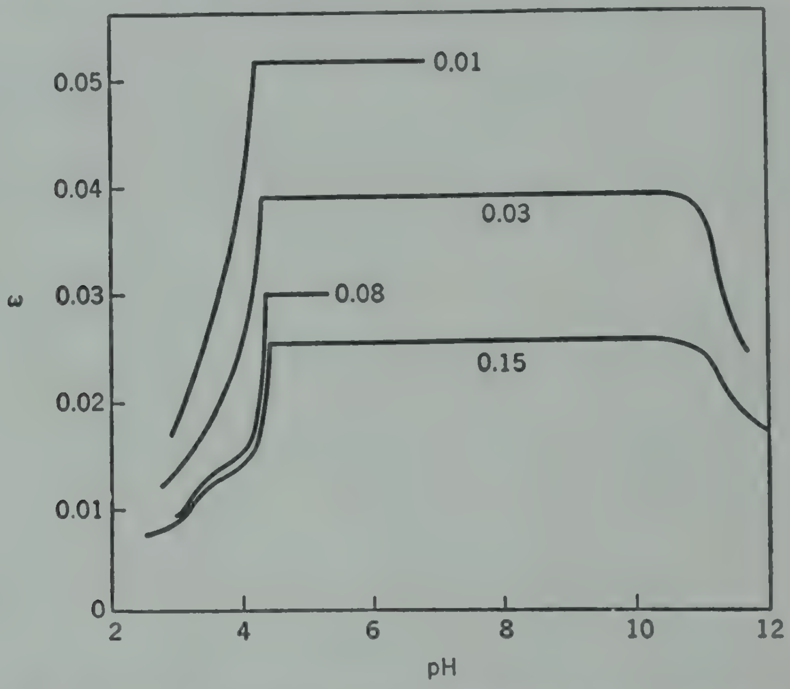

FIG. 7. The empirical electrostatic interaction factor ω as a function of pH at different ionic strengths. From Tanford *et al.* (108).

The breakdown of the simple "smeared site" model, with only one type of carboxyl group, in accounting for the titration curve of serum albumin is demonstrated in Fig. 7. The parameter ω is seen to suffer a sudden drop around pH 4.2. Independent evidence from viscosity and optical rotation measurements (109) indicates some deep-going configurational change in this pH region. The situation may not be unlike the one found for ferrihemoglobin (3). The formulation of a unified and consistent interpretation of the whole of the titration curve of serum albumin is one of the major problems in the theory of protein acid-base equilibria.

Acknowledgment

The authors would like to thank Dr. P. E. Wilcox for many helpful comments during he preparation of this manuscript.

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Chapter 3

Moving Boundary Electrophoresis—Theory

L. G. LONGSWORTH

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I. ELECTROPHORETIC METHODS

A. Microscopic Method

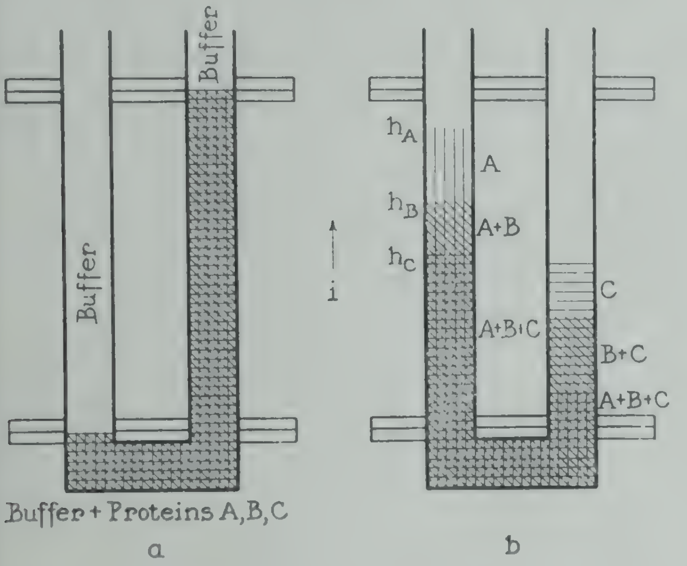
Electrophoresis is the movement of charged particles in solution under the influence of an electric field. As this definition suggests, if the particle is large enough its motion may be observed directly in the field of a microscope—a method for the study of electrophoresis that is treated in another chapter of this volume. Many charged particles, e.g. salt and protein ions, are not large enough, however, to be thus observed, and the following methods have been developed for their study.

B. Analytical Method

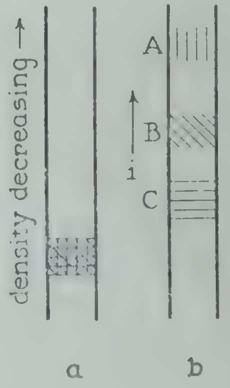
Early in the development of electrochemistry Kohlrausch (1) demonstrated theoretically, and it has been subsequently confirmed experimentally, that the passage of an electric current through a homogeneous conducting solution could not produce any changes in composition. Such changes could be expected, however, in regions in which discontinuities or concentration gradients existed initially. An unavoidable discontinuity in the circuit is the electrode-solution interface, and it was in the liquid in contact with the electrode that Hittorf first observed concentration changes on passage of the current (2). Although this, the analytical, method for the study of electrophoresis is not treated in detail in this volume it can be used to advantage in many problems. The development of materials containing a radioactive isotopic atom, and of quantitative procedures for the estimation of this activity, has enhanced the usefulness of the analytical method (3, 4).

C. Free Electrophoresis by the Moving Boundary Method

If, in addition to the phase boundaries represented by the electrode-solution interfaces, concentration gradients are also present initially in the liquid portion of the circuit, these are generally affected by the passage of the current. This is the basis of the moving boundary method for the study of electrophoresis (5-9). Suppose, for example, that an aqueous buffer solution in a U-shaped channel is homogeneous except for the presence of low concentrations of proteins in the region indicated by the crosshatching in Fig. 1, a. Here three proteins, A, B, and C, are represented by the vertical, diagonal, and horizontal crosshatching, respectively, and it is further assumed that the velocities of the protein ions in unit electric field, i.e. the mobilities, u, are in the order $u_A > u_B > u_C > 0$. After passage of current in the direction indicated, the protein distribution for this idealized case is shown in Fig. 1, b. Although not contributing very much to the electrical conductivity, the proteins generally increase appreciably the density and refractivity of the regions of the solution in which they are present. As will be described in the following chapter, optical methods are available for the recording of the refractive index, or its gradient, as a function of the height, h, in the channel and the levels at h_A, h_B, and h_C, Fig. 1, b, at which the concentrations of A, B and C, respectively, fall to zero with increasing height may thus be determined.



(1)



(2)

Fig. 1. Ideal free electrophoresis of a protein mixture.

Fig. 2. Ideal zone electrophoresis of a protein mixture.

It will be noted in Fig. 1, b that the passage of the current has effected a partial separation of the proteins. This is achieved, however, only if remixing of the separated portions by convective circulation is avoided. As the intensity of the crosshatching in Fig. 1, b suggests, the lighter solution is above the denser one at each boundary. This stabilization of the boundary system with respect to gravity by the density increments due to the components being separated is a characteristic feature of the moving boundary method. As applied to proteins and other macromolecular ions it is also known as the Tiselius method or that of free electrophoresis, terms that will be used interchangeably in this book. An equally characteristic feature of free electrophoresis is that complete separations are not possible. Thus, if current were passed until the most rapidly descending boundary of Fig. 1, b moved into the horizontal portion of the channel, remixing by gravity would ensue.

D. Zone Electrophoresis in Density Gradients

A variety of methods has been developed to permit complete separation as shown diagrammatically in Fig. 2. One involves the development, prior to electrophoresis, of a gravitationally stable, i.e. negative, density gradient in a vertical channel by appropriate mixing of the buffer solution and this same liquid to which a nonelectrolyte has been added in order to increase the density (10, 11, 12, 13). The density increments contributed by the solutes being separated are now undesirable and care must be exercised in introducing the solute zone, indicated by the crosshatching in Fig. 2, a, into the channel. The solute concentrations must also be such that positive density gradients do not arise as separation proceeds (Fig. 2, b).

If a pH gradient is superimposed on the density gradient prior to electrophoresis, what Kolin has termed isoelectric spectra may be obtained (14). If the pH increases with height, current must pass in the direction indicated in Fig. 2. At some level in the channel the pH is the isoelectric pH of one of the proteins. Any of the protein below this level initially migrates as cation, i.e. upward with the current, to this level, whereas that above the isoelectric level is present as anion and moves downward against the current. A protein is thus concentrated at the level at which it is isoelectric, and if the various species have different isoelectric pH values they will be concentrated at different levels. Here concentration accompanies separation.

Separation may also be accompanied by concentration if a variation of conductivity with height is superimposed on the density gradient, e.g. by arranging the column initially so that the concentrations of both the buffer salt and the nonelectrolyte decrease with increasing height. If, now, the mixture to be separated is placed in a zone near the top of the column and current passed so that the proteins descend, the leading edge of a separated zone moves progressively into regions of increasing conductivity and, at constant current, the zone contracts in height as it decelerates. What Kolin terms mobility spectra are obtained in this manner (14). The contraction of the zone does not imply increased resolving power, however, since the separation of successive zones decreases in proportion.

E. Zone Electrophoresis in Porous Media

In the channels of relatively large cross section used for free electrophoresis, any density gradient having a component other than vertical and negative tends to initiate circulation in accord with the laws of hydrodynamics. In a channel of circular cross section the flow decreases as the fourth power of the radius whereas the area is proportional to the square of this dimension. It is hardly surprising, therefore, that electrophoresis in capillary spaces has proved effective in removing the limitation imposed by the requirement for gravitational stability in free electrophoresis. As described elsewhere in this volume, a variety of materials is used to provide the capillary interstices, e.g. glass spheres, foam rubber, paper, intact and partially hydrolyzed starch, agar in concentrations from 0.13% to 2%, gelatin, etc. A variety of terms has been used to describe the process, electrophoresis in stabilized media, ionophoresis, ionography, electrochromatography, and the one adopted in this volume, zone electrophoresis. In contrast with electrophoresis in density gradients, that in capillary spaces can take place horizontally as well as vertically. Insofar as the disperse phase can be ignored, the theory of free electrophoresis presented in this chapter is applicable to the early stages of the zone technique since the partial separation of Fig. 1, b will be recognized as a step in the complete separation of Fig. 2, b.

II. MOVING BOUNDARIES FORMED BY STRONG ELECTROLYTES

A. The Hittorf Transference Number

Many of the concepts involved in electrophoresis may be illustrated with the aid of the Hittorf experiment shown in Fig. 3. Here a silver anode A and cathode C are immersed in a solution of silver nitrate (Fig. 3, a) containing g grams of salt per gram of water. That this solution is initially uniform in composition is shown by the heavy vertical line in the plot of concentration versus height (Fig. 3, b). If, now, f coulombs of electricity are passed at such a rate that the heating effect of the current does not cause circulation of the solution, the concentration changes resulting from the electrode processes, ion migration, and diffusion will be represented by the dashed curve of Fig. 3, b. After passage of the current the solution is drained slowly from the cell and collected in three portions corresponding roughly to those separated by the dotted lines in the figure. If each portion is then weighed and analyzed it will be observed, provided f has not been too large, that the concentration of the middle portion has not changed whereas the anode solution has become more concentrated and the cathode portion more dilute. If s_a and w_a are the weights of salt and water, respectively, in the anode portion, this mass of water originally contained gw_a grams of salt and has thus gained $(s_a - gw_a)$ grams on passage of the current. If S is the equivalent weight of silver nitrate the number of equivalents of this salt gained per Faraday equivalent, F, of electricity passed is the transference number $T = (s_a - gw_a)F\ fS$. Since the mass of silver deposited on the cathode is equal to that dissolved at the anode the total quantity of silver nitrate in solution remains unchanged and the shaded areas of Fig. 3, b are equal. Thus if s_c and w_c are the weights of salt and water in the cathode portion, a test of the relation $gw_c - s_c = s_a - gw_a$ affords a check on the experiment.

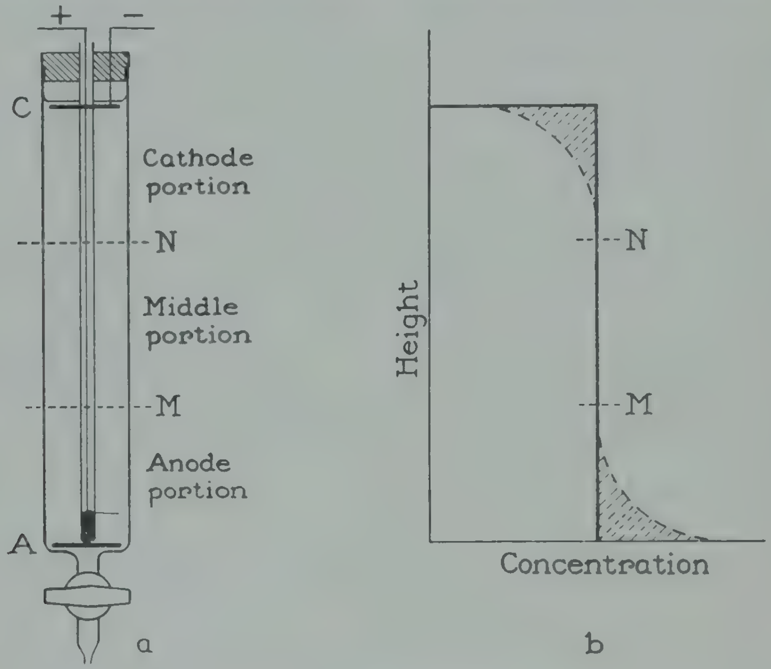


FIG. 3. The Hittorf experiment. See text for details.

The transference number as defined and measured by Hittorf involves no assumption as to the existence of ions in solution or the validity of Ohm's law. It remained for Arrhenius (15) to postulate the existence of charged particles in the solution and for Kohlrausch (16) to compute the velocities of these ions in the electric field. Silver nitrate and other strong electrolytes are now thought to be completely dissociated in aqueous solution. Since one equivalent of silver ion is formed at the anode per Faraday, whereas only T equivalents have been gained by this solution, it is clear that $1 - T$ equivalents have migrated upward through the planes at M and N, it being assumed that the current carried by the hydrogen and hydroxyl ions of the solvent is negligible. Thus the transference number may also be defined as the number of equivalents of an ion species that migrates through a plane in the body of a homogeneous solution on convection-free passage of one Faraday of electricity. In the present example $T_{NO_3} = T$ and $T_{Ag} = 1 - T$, but the experiment has afforded only one datum since the relation $T_{Ag} + T_{NO_3} = 1$ is an alternative expression for the electrical neutrality of the solution. If T varies with the concentration, the value obtained in a Hittorf experiment corresponds to the unchanged concentration of the middle portion.

It is important to note that the method of computation used by Hittorf gives the gain, or loss, of salt by a fixed mass of solvent. The ion migration is thus referred to the solvent as stationary and the mass of water on either side of a reference plane must, therefore, remain constant during electrophoresis. Owing to the volume changes that accompany the passage of the current, the planes M and N (Fig. 3) move slightly with respect to the apparatus. In transference number measurements on strong electrolytes with the moving boundary method, where the displacements refer to graduations on the channel, correction is made for these volume changes (7). In the free electrophoresis of proteins, however, the precision seldom warrants this correction, and it is not considered further.

The experiment of Fig. 3 is of practical significance for free electrophoresis since it emphasizes the need for a relatively large volume of solution between an electrode and the moving boundary channel. Thus the portion of the circuit shown in Figs. 1 and 2 is represented by the volume between the planes M and N of Fig. 3.

B. *Ion Mobilities and Ohm's Law*

If C_+ is the concentration of the silver ion in gram equivalents per liter of solution, the charge associated with this species is $FC_+/1000$ coulombs per milliliter. The factor $F/1000$ may be avoided, however, if the concentration is expressed directly as coulombs per cubic centimeter, and this will be designated c. The difference between the milliliter and the cubic centimeter, 0.00027%, is negligible in most instances. If the mobility, u_+, is the velocity of the silver ion in an electric field, E, of 1 volt per centimeter, the one-dimensional flow of this species in a channel of unit cross section corresponds to a current density, i_+, where

$$i_+ = u_+c_+E$$

A similar relation is valid for the nitrate ion and the total current density is

$$i = i_+ + i_- = (u_+c_+ + u_-c_-)E$$

Comparison with Ohm's law

$$i/\kappa = E$$

identifies the specific conductance, κ, as

$$\kappa = u_+c_+ + u_-c_- = F(u_+C_+ + u_-C_-)/1000$$

Since the concentration of silver nitrate is $C(= C_+ = -C_-)$ and the equivalent conductance, Λ, is 1000 κ/C

$$\Lambda = F(u_+ - u_-)$$

As implied here the ion concentration, the mobility, and the relative mobility, r, to be defined below are signed quantities, positive for cations and negative for anions. The current due to any ionic species, and the total current due to all ions, is taken as positive for a homogeneous phase.

With a constant current the time, t, required for F coulombs of electricity to pass is F/i and the number of equivalents of silver ion passing a plane in the body of the solution in this time is

$$T_+ = u_+E(c_+/F)(F/i) = i_+/i$$

The transference number is thus the fraction of the total current carried by the ion in question. In solutions of strong electrolytes it is always positive.

The relations developed here for a single binary salt may be generalized for a mixture of n ionic species.

$$\kappa = \Sigma c_j u_j \tag{1}$$

$$T_j = \frac{i_j}{i} = \frac{c_j u_j}{\kappa} = \frac{c_j u_j}{\Sigma c_j u_j} \tag{2}$$

$$\Sigma c_j = 0 \tag{3}$$

$$\Sigma T_j = 1 \tag{4}$$

Moreover, since the mobilities enter only through the transference number, where they always occur as a ratio, it is sufficient to specify the relative mobilities, r_j, that are obtained by dividing the set $u_1, u_2 \ldots u_n$ by the absolute magnitude of any member, say $|u_n|$. It is also convenient to consider the set, $u_1, u_2 \ldots u_n$ or $r_1, r_2 \ldots r_n$ as ordered from the most negative to the most positive. Unless otherwise indicated Σ represents summation over the n ion species.

In terms of the relative mobilities the transference number is unchanged in form

$$T_j = \frac{c_j r_j}{\Sigma c_j r_j} = \frac{c_j r_j}{\sigma} \tag{5}$$

where the denominator, σ, is the relative conductance.

C. The Boundary System

If remixing by convection is avoided, an initially sharp boundary between two solutions containing several ion species will generally split, on passage of an electric current, into a number of separate boundaries, each moving at a different rate. This was shown diagrammatically in Fig. 1 and is illustrated for an actual case in Fig. 4. Here the traced line is the variation of the refractive index gradient with height at the original boundary between the α and ζ solutions just prior to the passage of the current, whereas the boundaries that separated during electrophoresis are indicated by the five peaks, two that have moved anodically against the current, one stationary, and two that have moved cathodically. Such a set of separated boundaries is called a system, and a layer of homogeneous solution above or below a boundary is termed a phase. For the development of the general theory the phases of a system will be identified by Greek letters in order from the anode to the cathode side. It will also be necessary to assume that the relative mobilities are constant throughout the system, a good approximation for strong electrolytes at low concentrations, and are known. It will also be assumed that the compositions of the two solutions forming the original boundary, the so-called end solutions, are known. The end solution on the anode side is thus the α solution, whereas in the general case of n species that on the cathode side will be designated η.

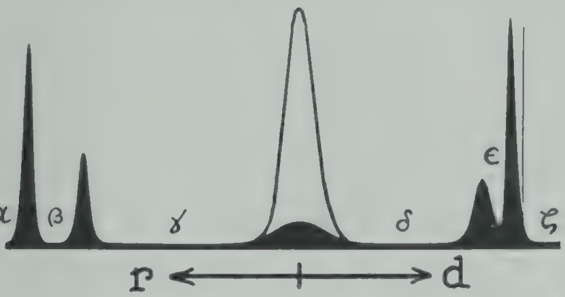


FIG. 4. A representative moving boundary system, 0.08 C LiCl, 0.08 C NaCl (α) : : 0.1 C $KBrO_3$, 0.1 C KIO_3 (ζ).

A separated boundary is thus identified by the two phases forming it, say β and γ, and the volume through which this boundary moves on passage of unit quantity of electricity will be designated $V^{\beta\gamma}$. The problem to be considered here is the composition of each of the new solutions formed by the splitting of the original boundary and the displacements of the separated boundaries. The solution of this problem is attributable to Dole (17).

The separated boundaries are not mathematical planes, as implied in Fig. 1, but the transition from one homogeneous phase to the other occurs within a finite height. In the case of strong electrolytes the principal factors controlling the composition gradients through a boundary are diffusion and variations of the field strength. In the case of weak electrolytes and proteins, pH changes, interactions between the ionic species, and electrophoretic heterogeneity are also factors. In many instances the concentration distribution in a separated boundary does not change as the boundary moves on passage of a constant current, and it is said to be in a steady state (18, 19). If the boundary spreads as it moves and this is due to diffusion, the position of the first moment or centroid of the refractive index gradient curve may be taken as the boundary position (20). The case in which the spreading is due to interactions within the boundary zone such that the centroids of individual constituents separate without generation of a homogeneous phase is considered later in this chapter under Reaction Boundaries (Section VII). The essential features of a separated boundary are that the solutions on either side be homogeneous and that the boundary does not split, i.e. form new homogeneous phases, on continued passage of the current.

D. The Moving Boundary Equation

Consider, for example, a separated boundary, *a* of Fig. 5, between the solutions α and β that moves against the current, i, through a volume $V^{\alpha\beta}$ to the position *b* on the passage of one Faraday equivalent of electricity.

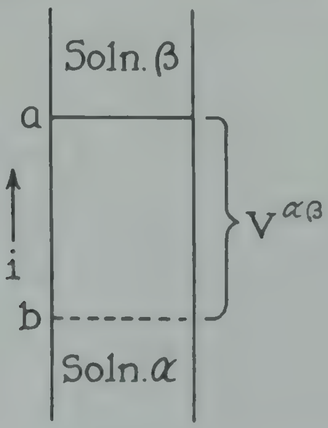


FIG. 5. Diagram for the moving boundary equation.

If C_j^α is the equivalent concentration of an anion j in the solution α and C_j^β its concentration in the β solution, the number of equivalents of this species present initially in the volume $V^{\alpha\beta}$ is $C_j^\alpha V^{\alpha\beta}$ and after passage of the current, $C_j^\beta V^{\alpha\beta}$. Moreover, a number of equivalents of the j ion equa to its transference number, T_j^β, in the β solution simultaneously enter this volume from above through the plane at a while T_j^α equivalents leave through the plane at b. Conservation of mass for the j ion in the volume $V^{\alpha\beta}$ then gives

$$T_j^\alpha - T_j^\beta = V^{\alpha\beta}(C_j^\alpha - C_j^\beta) \tag{6}$$

Similar considerations lead to the same relation when j is a cation if the concentrations are always taken with a sign corresponding to the charge on the ion. V is positive if the boundary moves with the current, negative if the migration is anodic. If C and c are in equivalents per liter and coulombs per cubic centimeter, respectively, V and v are in liters per Faraday equivalent and cubic centimeters per coulomb, respectively, since the transference number is dimensionless.

The following relation, obtained from equations (5) and (6)

$$\left(\frac{r_j}{\sigma^\alpha} - v^{\alpha\beta}\right) c_j^\alpha = \left(\frac{r_j}{\sigma^\beta} - v^{\alpha\beta}\right) c_j^\beta \tag{7}$$

and the relation obtained by elimination of the concentrations between equations (6) and (7), followed by multiplication of both sides by r_j,

$$\frac{T_j^\beta}{T_j^\alpha} = \frac{r_j - v^{\alpha\beta}\sigma^\alpha}{r_j - v^{\alpha\beta}\sigma^\beta} \tag{8}$$

will be useful in subsequent developments. For the case in which the boundary moves, $v^{\alpha\beta} \neq 0$, equation (7) may be rearranged, followed by multiplication of numerator and denominator by $v^{\alpha\beta}$, to give

$$c_j^\beta = \frac{(v^{\alpha\beta}\sigma^\beta)(v^{\alpha\beta}\sigma^\alpha - r_j)}{(v^{\alpha\beta}\sigma^\alpha)(v^{\alpha\beta}\sigma^\beta - r_j)}\, c_j^\alpha \tag{9}$$

This relation emphasizes the role that the $v\sigma$ products are to play in the development. Equations of the form of (9) can be written for each ionic species and for each moving boundary. If the two $v\sigma$ products characteristic of the $\alpha\beta$ boundary, i.e. $v^{\alpha\beta}\sigma^\alpha$ and $v^{\alpha\beta}\sigma^\beta$, are known, the composition of the β phase, and hence σ^β and $v^{\alpha\beta}$, can be computed, with the aid of equation (9), from the known properties of the end phase α. Similar equations are valid for each moving boundary in the system, and knowledge of the corresponding $v\sigma$ products thus permits evaluation of the composition of successive phases.

E. The Kohlrausch Regulating Function and the Stationary Boundary

A complication arises when the stationary boundary is reached. If this is the $\beta\gamma$ boundary then $v^{\beta\gamma} = 0$ and equation (7) becomes $c_j^\beta / c_j^\gamma = \sigma^\beta / \sigma^\gamma$, a relation containing two unknowns, c_j^γ and σ^γ. This is resolved with the aid of Kohlrausch's regulating function, ω, defined as

$$\omega = \Sigma \frac{c_j}{r_j} \tag{10}$$

If each member of the set of n equations represented by equation (7) is divided by r_j and the set added, recalling that $\Sigma c_j = 0$ and using equation (10),

$$\mathfrak{v}^{\alpha\beta}(\omega^\alpha - \omega^\beta) = 0$$

In the case of a moving boundary, $\mathfrak{v}^{\alpha\beta} \neq 0$ and $\omega^\alpha = \omega^\beta$. Consequently this function must have the same value in every phase on one side of the stationary boundary, regardless of the number of moving boundaries and phases, and must be that, ω^α or ω^η, of the end solution originally present in the regions now occupied by the new phases. If the $\beta\gamma$ boundary is stationary $c_j^\beta = c_j^\gamma \sigma^\beta / \sigma^\gamma$, and substitution in the expression for ω^β, ω^γ, equation (10), gives

$$\frac{c_j^\beta}{c_j^\gamma} = \frac{\sigma^\beta}{\sigma^\gamma} = \frac{\omega^\beta}{\omega^\gamma} \tag{11}$$

$$= \frac{\omega^\alpha}{\omega^\eta} = \rho \tag{12}$$

Thus the stationary boundary is one in which the concentrations of all species remain in the same relative proportions, the solution above this boundary being derivable from that below by dilution with water. The dilution factor ρ is given by the ratio of the regulating function for the end solutions and the condition for the absence of this boundary from the system is $\omega^\alpha = \omega^\eta$. Such a boundary is called a concentration boundary and in real systems its slight movement with respect to the solvent is a reflection of the approximate nature of the assumption that the relative mobilities are constant.

F. The Dole Polynomials

Returning to the evaluation of the $\mathfrak{v}\sigma$ products the next steps are to subtract unity from both sides of equation (8), compose fractions, multiply through by T_j^α and sum over all ion species to obtain

$$\Sigma T_j^\beta - \Sigma T_j^\alpha = \Sigma \frac{\mathfrak{v}^{\alpha\beta}(\sigma^\beta - \sigma^\alpha)}{r_j - \mathfrak{v}^{\alpha\beta}\sigma^\beta} T_j^\alpha = 0$$

Since the product $\mathfrak{v}^{\alpha\beta}(\sigma^\beta - \sigma^\alpha)$ is common to all terms in the summation and is not zero for a moving boundary it may be factored to give

$$\Sigma \frac{T_j^\alpha}{r_j - \mathfrak{v}^{\alpha\beta}\sigma^\beta} = 0 \tag{13}$$

This relation represents a polynomial in the unknown product $\mathfrak{v}^{\alpha\beta}\sigma^\beta$. If there are n species in the α solution the polynomial is of degree $n - 1$ and has a corresponding number of roots, one of which is $\mathfrak{v}^{\alpha\beta}\sigma^\beta$. As will be shown below all of these roots are real and equal, if arranged in order from the most negative to the most positive, to $v^{\alpha\beta}\sigma^{\beta}, v^{\beta\gamma}\sigma^{\gamma} \ldots v^{\zeta\eta}\sigma^{\eta}$, respectively. The corresponding relation for the other end solution, η, is

$$\Sigma \frac{T_j}{r_j - v^{\zeta\eta}\sigma^{\zeta}} = 0 \tag{14}$$

whose roots, similarly arranged, will be shown to be $v^{\alpha\beta}\sigma^{\alpha}, v^{\beta\gamma}\sigma^{\beta} \ldots v^{\zeta\eta}\sigma^{\zeta}$.

Writing equation (13) as

$$\Sigma \frac{T_j^{\alpha}}{r_j - x} = 0 \tag{15}$$

and identifying the roots as $x_1, x_2 \ldots x_{n-1}$, the following n linear equations in the n transference numbers of the α phase become

$$\begin{aligned} \Sigma T_j^{\alpha}/(r_j - x_1) &= 0 \\ \Sigma T_j^{\alpha}/(r_j - x_2) &= 0 \\ \cdots\cdots\cdots&\cdots \\ \Sigma T_j^{\alpha}/(r_j - x_{n-1}) &= 0 \\ \Sigma T_j^{\alpha} &= 1 \end{aligned}$$

Solution* of this system of equations for T_j^{α} is

---

* The solution, in determinant form, for a four-ion system is

$$T_1^{\alpha} = \frac{\begin{vmatrix} 0 & \frac{1}{r_2 - x_1} & \frac{1}{r_3 - x_1} & \frac{1}{r_4 - x_1} \\ 0 & \frac{1}{r_2 - x_2} & \frac{1}{r_3 - x_2} & \frac{1}{r_4 - x_2} \\ 0 & \frac{1}{r_2 - x_3} & \frac{1}{r_3 - x_3} & \frac{1}{r_4 - x_3} \\ 1 & 1 & 1 & 1 \end{vmatrix}}{\begin{vmatrix} \frac{1}{r_1 - x_1} & \frac{1}{r_2 - x_1} & \frac{1}{r_3 - x_1} & \frac{1}{r_4 - x_1} \\ \frac{1}{r_1 - x_2} & \frac{1}{r_2 - x_2} & \frac{1}{r_3 - x_2} & \frac{1}{r_4 - x_2} \\ \frac{1}{r_1 - x_3} & \frac{1}{r_2 - x_3} & \frac{1}{r_3 - x_3} & \frac{1}{r_4 - x_3} \\ 1 & 1 & 1 & 1 \end{vmatrix}}$$

Since three terms in the first column of the numerator are zero, this may be reduced to a third-order determinant by development in the minors of the first column. Moreover, three of the terms in the bottom row of the denominator can be made zero, without altering the value of the determinant, by subtracting each element of the first column from the remaining elements in that row. Development in the minors of the bottom row then leads to a single third-order determinant. On composition of the fractions in each term of the reduced determinant in the denominator, it will be noted that

$$T_j^\alpha = \frac{(r_j - x_1)(r_j - x_2)\ \ldots\ (r_j - x_{n-1})}{(r_j - r_1)(r_j - r_2)\ \ldots\ (r_j - r_n)} \tag{16}$$

the zero factor, $r_j - r_j$, being absent from the denominator. Identifying the roots of equation (14) as $y_1, y_2 \ldots y_{n-1}$ and proceeding similarly

$$T_j^\eta = \frac{(r_j - y_1)(r_j - y_2)\ \ldots\ (r_j - y_{n-1})}{(r_j - r_1)(r_j - r_2)\ \ldots\ (r_j - r_n)} \tag{17}$$

The denominators of equations (16) and (17) are identical and may be eliminated to give

$$\frac{T_j^\alpha}{T_j^\eta} = \frac{(r_j - x_1)(r_j - x_2)\ \ldots\ (r_j - x_{n-1})}{(r_j - y_1)(r_j - y_2)\ \ldots\ (r_j - y_{n-1})}$$

When this is compared with the following identity, obtained with the aid of equation (8),

$$\frac{T_j^\alpha}{T_j^\eta} = \frac{T_j^\alpha}{T_j^\beta} \cdot \frac{T_j^\beta}{T_j^\gamma} \cdots \frac{T_j^\zeta}{T_j^\eta} = \frac{(r_j - v^{\alpha\beta}\sigma^\beta)(r_j - v^{\beta\gamma}\sigma^\gamma)\ \ldots\ (r_j - v^{\zeta\eta}\sigma^\eta)}{(r_j - v^{\alpha\beta}\sigma^\alpha)(r_j - v^{\beta\gamma}\sigma^\beta)\ \ldots\ (r_j - v^{\zeta\eta}\sigma^\zeta)}$$

it is noted that the roots of the polynomials are, in fact, the desired $v\sigma$ products.

G. Identification of the Roots of the Polynomial

Although it is a consequence of the notation that $v^{\alpha\beta} < v^{\beta\gamma} < v^{\gamma\delta} \ldots$, it is not evident that the $v\sigma$ products follow the same sequence. A plot of equation (15), considered as a function of x and specialized in Fig. 6 for a mixture of three anions 1, 2, 3 and two cations 4, 5, will aid in the identification of the roots. Since $f(x)$ approaches $-\infty$ as x approaches r_1 from the less negative side and $+\infty$ as it approaches r_2 from the more negative side, $f(x)$ must cross the axis, i.e. have a root somewhere between r_1 and r_2. Similarly the other roots must occur between successive pairs of r values, that between the least negative r and the least positive r being at the origin, since $x = 0$ is a root of the polynomial. This becomes clear on substitution of $x = 0$ in equation (15) and noting that $\Sigma c_j^\alpha = 0$. In like manner the roots of equation (14) occur in the same successive intervals.

Since the transference numbers and relative conductances are positive,

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$\frac{1}{r_1 - x_1}, \frac{1}{r_1 - x_2}$ and $\frac{1}{r_1 - x_3}$ are factors of the first, second, and third rows, respectively, whereas $(r_1 - r_2)$, $(r_1 - r_3)$ and $(r_1 - r_4)$ are factors of the first, second, and third columns, respectively. The determinant remaining after removal of these factors is then identical with that in the numerator, thus canceling it and yielding

$$T_1^\alpha = \frac{(r_1 - x_1)(r_1 - x_2)(r_1 - x_3)}{(r_1 - r_2)(r_1 - r_3)(r_1 - r_4)}$$

to be compared with equation (16).

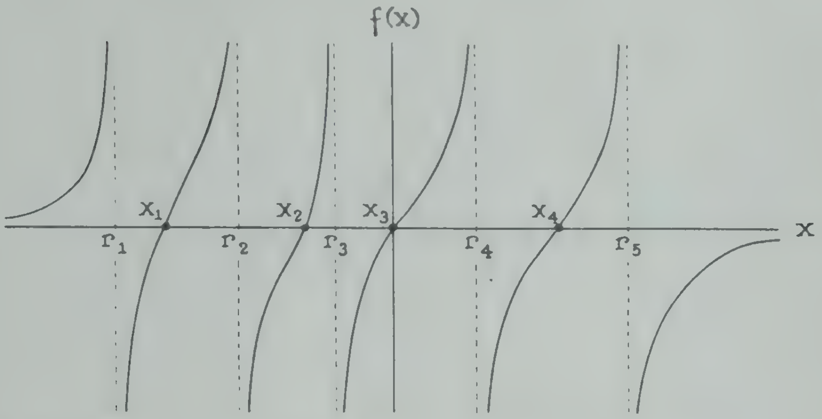


FIG. 6. Graph of equation (15) for a mixture of three anions and two cations.

equation (8) for $j = 1$ tells us that $v^{\alpha\beta}\sigma^{\alpha}$ and $v^{\alpha\beta}\sigma^{\beta}$ are on the same side of r_1. Moreover, since $v^{\alpha\beta}\sigma^{\beta} < v^{\beta\gamma}\sigma^{\beta}$ and $v^{\beta\gamma}\sigma^{\gamma} < v^{\gamma\delta}\sigma^{\gamma}$ both $v^{\beta\gamma}\sigma^{\beta}$ and $v^{\beta\gamma}\sigma^{\gamma}$ must be in the next more positive interval, i.e. between r_2 and r_3, whereas $v^{\alpha\beta}\sigma^{\alpha}$ and $v^{\alpha\beta}\sigma^{\beta}$ are in the interval between r_1 and r_2. Thus the sequence suggested above is confirmed.

H. Number of Boundaries

From the foregoing analysis a system of n ions will generally form $n - 1$ boundaries. If there are p anions and q cations, $p - 1$ boundaries move against the current, one is stationary and $q - 1$ have positive velocities. Less than $n - 1$ boundaries may be expected if the $v\sigma$ products for a moving boundary are equal, i.e. $x_i = y_i$, since the concentration of each species is identical, by equation (9), in adjoining phases and no gradients exist. As already noted the condition for the absence of the stationary boundary is $\omega^{\alpha} = \omega^{\eta}$.

The absence of a species from an end phase does not reduce the number of boundaries. In such a case the polynomial for this solution is reduced in degree but the $v\sigma$ product thereby lost is obtained directly from the fact that the absent ion must disappear across a moving boundary. If the g ion is present in the α solution but absent from the β phase, equation (7) reduces to

$$v^{\alpha\beta}\sigma^{\alpha} = r_{g} \tag{18}$$

for this ion and to

$$c_j^{\beta} = \frac{v^{\alpha\beta}\sigma^{\beta}(r_g - r_j)}{r_g(v^{\alpha\beta}\sigma^{\beta} - r_j)}\, c_j^{\alpha} \tag{19}$$

for all other species. Protein boundaries are of this type. It is also possible for two species to disappear in opposite directions across a moving boundary. If h and g are absent from the α and β phases, respectively, $v^{\alpha\beta}\sigma^{\alpha} = r_g$,

$v^{\alpha\beta}\sigma^{\beta} = r_{\mathrm{h}}$ and

$$c_j^{\beta} = \frac{r_{\mathrm{h}}(r_{\mathrm{g}} - r_j)}{r_{\mathrm{g}}(r_{\mathrm{h}} - r_j)}\, c_j^{\alpha} \tag{20}$$

The disappearance of two species in the same direction across a moving boundary implies equality of their mobilities and thus they are not different ions in the sense of this treatment. Equations (19) and (20) illustrate a general property of moving boundaries. No two species having different mobilities can remain in the same relative proportions across a moving boundary. The importance, in electrophoretic analyses, of these superimposed gradients at a boundary across which a protein ion disappears was first emphasized by Svensson (21).

The polynomials actually used for computation are obtained from equations (13) and (14) by use of equation (5) and elimination of any one of the concentrations, say c_n, with the aid of the condition for electrical neutrality, $\Sigma c_j = 0$,

$$\frac{r_1 - r_n}{r_1 - x}\, c_1^{\alpha} + \frac{r_2 - r_n}{r_2 - x}\, c_2^{\alpha} + \cdots \frac{r_{n-1} - r_n}{r_{n-1} - x}\, c_{n-1}^{\alpha} = 0 \tag{21}$$

$$\frac{r_1 - r_n}{r_1 - y}\, c_1^{\gamma} + \frac{r_2 - r_n}{r_2 - y}\, c_2^{\gamma} + \ldots \frac{r_{n-1} - r_n}{r_{n-1} - y}\, c_{n-1}^{\gamma} = 0 \tag{22}$$

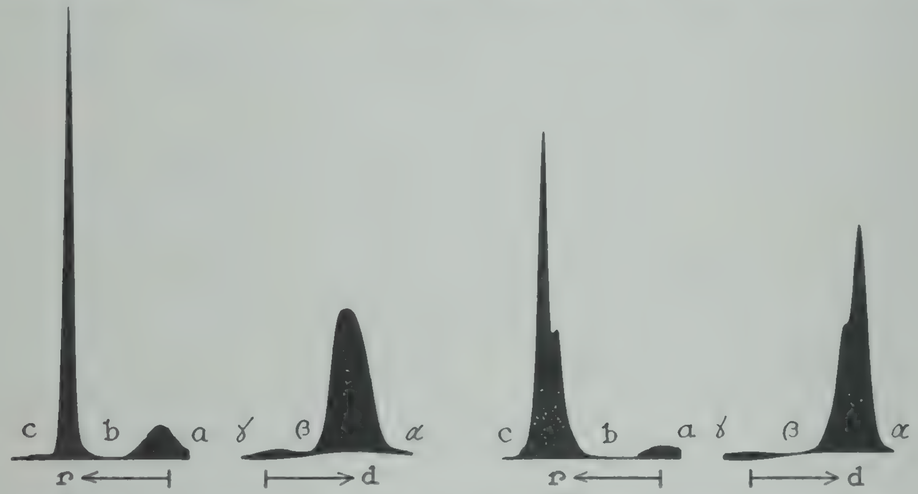
Some useful approximations are given later as equations (29) and (30).

III. Electrophoresis of Proteins

A. *Ideal Electrophoresis*

In the free electrophoresis of a protein solution the initial boundary in each side of the U-shaped channel is generally formed between the two solutions that result from the dialysis of the protein solution against a large volume of an appropriate buffer solution. The diffusible buffer ions are then present in the end solutions at concentrations corresponding to a Donnan equilibrium. Moreover, within the pH-stability range of most proteins the mass per unit net charge, i.e. the equivalent weight of the macromolecular ion, is large in comparison with that of the buffer ions. Thus a concentration of protein that increases appreciably the refractivity and density of the solution does not contribute much to the conductivity nor compete very successfully with the buffer electrolyte for the control of the pH. Ideal free electrophoresis may thus be visualized as the limiting case in which the conductance and pH effects due to the protein are negligible. The buffer electrolytes are present throughout the channel in sufficient concentrations to provide a constant pH and, on passage of the current, a uniform electric field that is not altered by the migration of protein into, or out of, a region. The regulating function is then identical for the end solutions, no concentration gradients remain at the level of the original boundary and at a moving boundary there are no gradients of species other than the protein ion disappearing therein. Moreover, the pattern of the boundary system in one side of the channel is the mirror image of that in the other side and the concentration distribution through a boundary is determined solely by diffusion.

At low protein concentrations, and pH values in the neutral range, actual systems approach, but do not attain, the ideal behavior outlined above. The most frequently observed departures are illustrated by the patterns of Fig. 7 for a typical case (22) and are (a) the presence of the concentration boundaries $\beta\gamma$ and ab with the refractive index difference $n^a - n^b > n^\beta - n^\gamma$, (b) $v^{bc} > v^{\alpha\beta}$ and (c) the spreading of the bc boundary is less than diffusion alone would produce whereas $\alpha\beta$ has spread faster than can be accounted for by this effect.



1.36% ovalbumin as cations in 0.1N NaAc at pH 3.93
a

1.30% ovalbumin as anions in 0.1N NaAc at pH 5.34
b

FIG. 7. Electrophoretic patterns of ovalbumin at pH 3.93 (a) and pH 5.34 (b).

B. Notation

Although the initial boundary in each side of the channel is formed between the same solutions, in one side the current flows from the buffer to the protein solution whereas this flow is in the opposite direction in the other side. Thus a given pair of end solutions develops, on passage of the current, a pair of moving boundary systems and a notation that distinguishes between these conjugate systems is desirable. In work with real systems it is also helpful to distinguish between anions and cations and to identify not only the direction a boundary moves with respect to the current but also with respect to gravity. To this end the notation used thus far will be modified and extended as follows.

A, B, C . . . will refer to cations whose mobilities are in the order $u_A > u_B > u_C$ . . . while R, S, T . . . indicate anions for which $|u_R| > |u_S| > |u_T|$ . . . . The initial boundary is indicated by separating with a dash, —, the symbols for the ions in the end solutions. In the system that develops on passage of the current a moving boundary is denoted by an arrow, →, while a double colon, ::, will be used for the concentration boundary remaining near the level of the original boundary. As before, the current is taken as flowing from left to right and the solutions meeting at the boundaries will be denoted by lower case letters in the order of decreasing density. On that side of the channel in which the boundaries descend Greek letters will be used whereas italic letters will be used for the other system of the pair.

Thus the system represented by the left-hand pattern of Fig. 7, a is $\mathrm{ABR}(a)::\mathrm{ABR}(b) \rightarrow \mathrm{AR}(c)$ and for the right hand pattern $\mathrm{AR}(\gamma)::\mathrm{AR}(\beta) \rightarrow \mathrm{ABR}(\alpha)$, where A, B, and R refer to sodium, protein, and acetate, respectively. At the higher pH, Fig. 7, b, the left-hand pattern corresponds to $\mathrm{AR}(c) \leftarrow \mathrm{ARS}(b)::\mathrm{ARS}(a)$ and that on the right to $\mathrm{ARS}(\alpha) \leftarrow \mathrm{AR}(\beta)::\mathrm{AR}(\gamma)$, where S now indicates the protein. The end protein solution is always α or a.

C. Electrophoresis of a Single Protein

In the systems of Fig. 7 the protein ion disappears across the $\alpha\beta$ boundary from an end solution, α, of known conductance, κ^α. Equation (18) becomes u_B (or u_S) $= v^{\alpha\beta}\kappa^\alpha$, in which $v^{\alpha\beta}$ is the displacement, in milliliters, of the first moment of the gradient curve for the boundary per coulomb of electricity passed. At the *bc* boundary the protein ion disappears from a solution, *b*, that has been formed by the passage of the current and the relation $u = v^{bc}\kappa^b$ is not of immediate value.

In the present example the dilution factor for the concentration boundaries, equation (12), becomes

$$\rho = \frac{\omega^\alpha}{\omega^\gamma} = 1 + \left(\frac{r_B - r_R}{r_B}\,\frac{r_A}{r_A - r_R} - \frac{1}{2}\right)\frac{C_B{}^\alpha}{C_A{}^\gamma}$$

where the first term in the expansion of the Donnan equation (23, 24) has been used to give the composition of the protein solution

$$C_A{}^\alpha = C_A{}^\gamma - \tfrac{1}{2}\,C_B{}^\alpha \qquad C_R{}^\alpha = C_R{}^\gamma - \tfrac{1}{2}\,C_B{}^\alpha$$

The difference in refractive index across the buffer concentration boundary

$\beta\gamma$ is then

$$\begin{aligned} n^\beta - n^\gamma &= K_{\mathrm{AR}}(C_{\mathrm{A}}{}^\beta - C_{\mathrm{A}}{}^\gamma) = K_{\mathrm{AR}}(\rho - 1)C_{\mathrm{A}}{}^\gamma \\ &= K_{\mathrm{AR}}\left(\frac{r_{\mathrm{B}} - r_{\mathrm{R}}}{r_{\mathrm{B}}}\,\frac{r_{\mathrm{A}}}{r_{\mathrm{A}} - r_{\mathrm{R}}} - \frac{1}{2}\right)C_{\mathrm{B}}{}^\alpha \end{aligned} \tag{23}$$

where K_{AR} is the increment of refractive index per equivalent of sodium acetate, AR. At the protein concentration boundary, *ab*,

$$\begin{aligned} n^{\mathrm{a}} - n^{\mathrm{b}} &= K_{\mathrm{AR}}(C_{\mathrm{A}}{}^{\mathrm{a}} - C_{\mathrm{A}}{}^{\mathrm{b}}) + K_{\mathrm{BR}}(C_{\mathrm{B}}{}^{\mathrm{a}} - C_{\mathrm{B}}{}^{\mathrm{b}}) \\ &= [K_{\mathrm{AR}}C_{\mathrm{A}}{}^\gamma + (K_{\mathrm{BR}} - \tfrac{1}{2}K_{\mathrm{AR}})C_{\mathrm{B}}{}^\alpha](1 - 1/\rho) \end{aligned} \tag{24}$$

where the equivalent refraction K_{BR} is that of the protein salt, ovalbumin acetate in the present example. Since $r_{\mathrm{B}} = v^{\alpha\beta}\sigma^\alpha = v^{\mathrm{bc}}\sigma^{\mathrm{b}}$, equation (18), and $\sigma^{\mathrm{a}}/\sigma^{\mathrm{b}} = \rho$, equation (11), the ratio of the displacements of the rising and descending boundaries is

$$v^{\mathrm{bc}}/v^{\alpha\beta} = \sigma^\alpha/\sigma^{\mathrm{b}} = \rho\sigma^\alpha/\sigma^{\mathrm{a}} = \rho \tag{25}$$

In Table I a comparison of the theory with experiment is made for three concentrations of protein at pH 3.92 and one concentration at pH 5.34. The relative mobilities and equivalent refractions used in the computations are to be found in Table II of this chapter and Table I of the next chapter. The equivalent concentration of protein is given by the relation $C_{\mathrm{B}}{}^\alpha = 10pe$ where e is the number of equivalents of hydrogen ion required to bring one gram of the isoelectric protein to the pH, and at the ionic strength, of the experiment and is taken from the titration data of Cannan and associates (25).

TABLE I

Comparison of Theory with Experiment in the Electrophoresis of Ovalbumin

	$r_{\mathrm{A}} = 1.000$	$r_{\mathrm{B}} = 0.1392$	$r_{\mathrm{R}} = -0.7875$	$r_{\mathrm{S}} = -0.1270$
pH^α	5.34	3.92	3.92	3.92
p (gm. protein/100 ml.)	1.29	0.64	1.36	2.74
$C_{\mathrm{B}}{}^\alpha$ or $C_{\mathrm{S}}{}^\alpha$	−0.00257	0.00150	0.00318	0.00641
$n^\beta - n^\gamma$ (equation 23)	0.000095	0.000060	0.000127	0.000255
(observed)	0.00007_5	0.00004_8	0.00012_3	0.00024_7
$n^{\mathrm{a}} - n^{\mathrm{b}}$ (equation 24)	0.000248	0.000114	0.000351	0.001085
(observed)	0.00016_5	0.00015_8	0.00049_4	0.00149_5
(equation 28)	0.000183	0.000146	0.000476	0.001509
pH^{b} (equation 26)	5.30	3.90	3.88	3.83
$v^{\mathrm{bc}}/v^{\alpha\beta}$ (equation 25)	1.085	1.048	1.103	1.207
(observed)	1.058	1.091	1.180	1.333
(equation 27)	1.056	1.078	1.166	1.341

TABLE II

BUFFER ION MOBILITIES AND SALT REFRACTIVITIES AT 0°C. $\lambda = 5780$ Å AND 0.1 C

Ion R	$u \times 10^5$ (35) $\text{cm}^2\ \text{volt}^{-1}\ \text{sec}^{-1}$	K_{NaR} (22) $\Delta n/C$
Chloride	−37.0	0.0112_0
Phosphate (dibasic)	−20.2	0.0148_4*
Acetate	−16.6	0.0123_5*
Glycinate—$H_2NCH_2COO^-$	−15.5	0.0174_6
Lactate	−13.1	0.0191_4*
Phosphate (monobasic)	−12.0	0.0172_0*
Borate—BO_2^-	−11.5	0.0207_9
Diethylbarbiturate	−10.2	0.0405_5
Cacodylate	−10.1	0.0242_3
A		K_{ACl}
Triethanolamine H	9.1	
Diethanolamine H	11.4	
Glycinium—$^+H_3NCH_2COOH$	$12._4$	0.0200†
Sodium	22.2	0.0112_0
Hydrogen	217.2	0.0089_4*

* $C = 0.2$ equivalents per liter solution.

† Uncorrected for hydrolysis.

In Table I it will be noted that the relations given above predict adequately the refractive index difference at the buffer concentration boundary $\beta\gamma$, but not that at the *ab* boundary nor the ratio of the displacements of the moving boundaries. This illustrates a limitation of the strong electrolyte theory when applied to a species, the protein ion, whose mobility is pH dependent. Thus far no account has been taken of the weak buffer acid, HR, that is present at a relatively high concentration at pH 3.92. If this component were diluted at the $\beta\gamma$ boundary in the same proportion as the ions, equation (23) would not have been in accord with experiment. This suggests that the weak buffer acid be considered one component of a mixed solvent whose concentration remains invariant during electrophoresis. Insofar as the buffer capacity of the protein is negligible in comparison with that of the acetic acid this is equivalent to the assumption that is made in the moving boundary theory for weak electrolytes, namely, that weak acid is conserved at each level in the channel. It is a consequence of this assumption, however, that the pH values of the β and b solutions differ from pH^α since for small variations in composition

$$\text{pH} = \text{constant} + \log (C_{\text{AR}}/C_{\text{HR}})$$

Consequently

$$\text{pH}^{\text{a}} - \text{pH}^{\text{b}} = \log \rho \tag{26}$$

and values computed with the aid of equation (26) are given in Table I.

At pH 3.92 and $\Gamma/2 = 0.1$, $(1/u_{\text{B}})\, du_{\text{B}}/d\text{pH} = -1.36$ for ovalbumin (25a) and the velocity of the protein ion in the b solution is higher than in the a solution not only because of the higher field strength, $E^{\text{b}}/E^{\text{a}} = \sigma^{\text{a}}/\sigma^{\text{b}} = \rho$, but also because of the lower pH. Correction for the pH effect on the boundary displacement is made by increasing v^{bc} in the ratio of the protein ion mobility in the b and a solutions.

$$v^{\text{bc}}/v^{\alpha\beta} = \rho u_{\text{B}}{}^{\text{b}}/u_{\text{B}}{}^{\text{a}} = \rho[1 - (1/u_{\text{B}})(du_{\text{B}}/d\text{pH}) \log \rho] \tag{27}$$

The improved agreement with the observed values (Table I) is evidence for the validity of the assumption that the concentration of weak buffer acid remains essentially uniform throughout the system.

To correct for the pH effect at the ab boundary $C_{\text{B}}{}^{\text{b}}$ of equation (24) is reduced by the factor $u_{\text{B}}{}^{\text{a}}/u_{\text{B}}{}^{\text{b}}$

$$n^{\text{a}} - n^{\text{b}} = K_{\text{AR}}(C_{\text{A}}{}^{\text{a}} - C_{\text{A}}{}^{\text{b}}) + K_{\text{BR}}(C_{\text{B}}{}^{\text{a}} - C_{\text{B}}{}^{\text{b}} u_{\text{B}}{}^{\text{a}}/u_{\text{B}}{}^{\text{b}}) \tag{28}$$

and the computed refraction increments are then in agreement with observation. In a more exact treatment of the problem the concentrations of the buffer ions would be adjusted to preserve electrical neutrality, but the effect of this adjustment is small.

At pH 5.34, where the protein is an anion and $(1/u_{\text{S}})du_{\text{S}}/d\text{pH} = 0.78$, the lower pH in the b solution reduces the magnitude of its mobility and the pH effect partially compensates for the conductance effect. Conjugate patterns are said to be enantiographic if they are mirror images of each other. The departures from enantiography in Fig. 7 are thus seen to be due both to the conductance changes predicted by the theory for strong electrolytes, which assumes the relative mobilities to be constant, and to pH changes which partially invalidate this assumption as regards the protein ion. In buffers of the uncharged acid type, e.g. acetic acid-acetate, the patterns tend to be more nearly enantiographic above the isoelectric pH of the protein than below, whereas the converse is true for buffers of the uncharged base type, e.g. glycine-glycinium.

An additional deviation from enantiography illustrated in Fig. 7 is the relative sharpness of the rising boundary. If the conductivity is lower behind a moving boundary than ahead of it, the ions in the trailing layers of the boundary are in a stronger electric field than those in leading layers and thus tend to overtake the ions in the van. Consequently such a boundary spreads less rapidly than from diffusion alone and is thus sharper than if the conductivity change aids diffusion. In the general case where a protein ion C disappears across the rising boundary *bc* and the descending boundary $\beta\gamma$, then $r_C = v^{\beta\gamma}\sigma^{\beta} = v^{bc}\sigma^{b}$ whereas the corresponding root of the Dole polynomial for the $\alpha(a)$ solution is $v^{\beta\gamma}\sigma^{\gamma} = v^{bc}\sigma^{c}$. If, as is usually the case, the mobilities of the protein ions are intermediate between those of the buffer ions, Fig. 6 indicates that the root is greater than r_C. Consequently $\sigma^{\beta} < \sigma^{\gamma}$, $\sigma^{b} < \sigma^{c}$ and the conductivity increases with the height at each moving boundary in both sides of the channel, a result first obtained by Svensson (21). A rising boundary thus tends to be sharper than the corresponding descending one. If, however, the mobility of the protein ion is greater in magnitude than that of the buffer ion of the same sign, the conductivity change is reversed and the rising boundary then becomes, in the absence of a pH effect, the diffuse one.

As has been noted in connection with Fig. 7, a the pH change may enhance the sharpening and spreading effects of the conductivity changes at the rising and descending boundaries, respectively, or, as in Fig. 7, b the pH effect may partially counteract the conductivity effect. In work with weak electrolytes of low molecular weight boundaries are sometimes encountered in which the pH effect outweighs the conductivity change and the sharp boundary moves into the solution with the low conductance. In the case of a 0.3% solution of ovalbumin in 0.01 *C* sodium diethylbarbiturate at pH 8.6 the conductivity effect is so small, the protein and diethylbarbiturate ions having the similar mobilities of -10.8×10^{-5} and -11.9×10^{-5}, respectively, that the pH effect dominates, causing the descending boundary to be the sharp one. The enantiography of the conjugate patterns thus frequently affords qualitative insight into the mobility relations of the components.

D. Electrophoretic Analysis of Protein Mixtures

In the three-ion systems thus far considered the Dole polynomials were of the first degree or less and explicit relations were obtained for comparison with experiment. Moreover, the principal interest in three-ion systems is for mobility determinations and, as will be described later in this chapter, measurements of heterogeneity. With mixtures of two or more proteins, i.e. ion systems of four or more, the problem of most interest is the extent to which the *apparent* composition is the true one. In obtaining the apparent composition, the superimposed gradients at a moving boundary are ignored and the relative concentration of a protein component on the basis of refractive index is taken as the ratio of the area of the peak in the pattern due to this component to the sum of the areas due to all components.

In the preparation of Table III, pattern characteristics have been computed for hypothetical systems and then analyzed in the conventional manner, assuming ideal behavior (22). Comparison of the results thus obtained with the postulated properties then indicate the errors in the apparent values. The hypothetical mixture consisted of two protein anions, S and T, each at a concentration of 1% in 0.1 C buffers with mobilities, relative to that of the sodium ion as unity, of $r_S = -0.3$ and $r_T = -0.15$ and equivalent concentrations $C_S = -0.0036$ and $C_T = -0.0018$. The buffer ion mobilities and salt refractions used in the computation have been taken from Table II and since the end solutions contain two and four ion species, respectively, the Dole polynomials reduce to a single quadratic function. Insofar as it is permissible to lump the serum globulins together as a single component T, the postulated system could represent serum, diluted with two to three volumes of buffer, at pH 8.6 in which the albumin-globulin ratio is unity.

In Table III the buffer anions are listed in the order of increasing negative mobility and it is at once apparent that the pattern enantiography increases as the differences between the protein mobilities and the mobility of the buffer ion of the same sign decrease. The apparent percentage of fast protein, columns 2 and 3, exceeds the postulated value of 50% in both patterns, but the descending boundaries yield the best value. As shown by Svensson (26) for a single protein the displacement ratios for the fast boundary, column 4, and the slow one, column 5, increase as $|r_A|$ increases. In a given buffer this ratio is greater for the slow than for the fast boundary and is in accord, therefore, with experiment. In an analysis of twenty-five human plasmas, diluted 1 to 3 in a 0.1 C sodium diethylbarbiturate buffer at pH 8.6, Dole (27) obtained 1.06 as the average displacement ratio for the albumin boundary and 1.13 for the globulin boundaries.

If the mobility of the slow protein, T, is computed from the displacement of the slow, descending boundary $\beta\gamma$ and the known conductance σ^α of the protein solution, the relative values of column 6 are obtained. Comparison of $r^{\beta\gamma}\sigma^\alpha$ with the postulated value of -0.15 indicates the errors that are made in the conventional evaluation of this mobility. The errors are less, however, than if v^{bc} is used and thus in all respects the pattern of the descending boundaries warrants most weight in the analysis. The magnitudes of the concentration boundaries are suggested by the entries in columns 7 and 8 of Table III.

At a boundary across which the j protein disappears the superimposed gradients due to nondisappearing species are, however, not effects of higher order that approach zero asymptotically as the concentration of j is decreased. Thus the computed apparent composition extrapolates to a value slightly different from the assumed one as the ratio of the protein

TABLE III

SOLUTION OF THE DOLE POLYNOMIALS FOR A MIXTURE OF THE PROTEINS S AND T, EACH AT A CONCENTRATION OF 1% IN 0.1 *C* SODIUM BUFFER[a]

1	2 Apparent composition (% S)	3	4	5	6	7 Concn. boundaries ($\Delta n \times 10^6$)	8
Buffer anion (R)	Rising	Descending	$\upsilon^{cd}/\upsilon^{\alpha\beta}$	$\upsilon^{bc}/\upsilon^{\beta\gamma}$	$-\upsilon^{\beta\gamma}\sigma^{\alpha}$	Buffer-$\gamma\delta$	Protein-ab
Diethylbarbiturate	52.49	50.52	1.037	1.085	0.1471	268	473
Lactate	53.47	50.93	1.044	1.111	.1459	155	412
Glycinate	54.16	51.50	1.051	1.138	.1444	166	473
Acetate	54.69	51.81	1.055	1.150	.1437	126	458
Phosphate (dibasic)	55.67	52.59	1.059	1.189	.1411	176	543
Chloride	57.12	53.44	1.084	1.257	.1381	176	649

Conjugate systems: $\text{ARST}(\alpha) \leftarrow \text{ART}(\beta) \leftarrow \text{AR}(\gamma) :: \text{AR}(\delta)$
$\text{AR}(d) \leftarrow \text{ASR}(c) \leftarrow \text{ARST}(b) :: \text{ARST}(a)$

---

[a] Assume: $r_A = r_{Na} = 1.000$; r_R (Table II); $r_S = -0.3$; $r_T = -0.15$; $C_S = -0.0036$; $C_T = -0.0018$

concentration to that of buffer salt approaches zero. If A and R are the buffer ions and j is a protein ion of the same sign as R, then for sufficiently low protein concentrations the roots of the Dole polynomial are [cf. reference (28)]

$$x_j \simeq r_j - \frac{(r_A - r_j)(r_j - r_R)}{r_A - r_R} \frac{C_j}{C_R} \tag{29}$$

In the four-ion system of Table III the extrapolated apparent fraction, $F°_S$, of S is

$$F°_S = \frac{(1 - M_S)}{1 - M_S f_S - M_T f_T} f_S \tag{30}$$

where f_S and f_T are the true fractions and

$$M_j = \frac{K_{AR} r_R (r_j - r_A)}{K_{Aj} r_j (r_R - r_A)}$$

Thus an extrapolated apparent value of 49.65% S is obtained in the diethylbarbiturate buffer and 49.8% in the others.

In most instances this difference between the extrapolated and assumed value is not sufficient, however, to invalidate the use of a plot of apparent composition versus the protein:salt ratio for extrapolation to the true composition at zero value for the ratio. The experiments may be designed to permit extrapolation of the apparent composition (a) to zero total protein concentration at constant ionic strength, (b) to zero reciprocal ionic strength at constant protein concentration, or (c), if neither protein nor salt concentration is held constant, to a zero value for the ratio. Of the three methods of extrapolation (a) is preferable since the relative mobilities are less sensitive to variations of protein concentration than to that of salt and interference optics permit the study of dilute solutions.

IV. Weak-Electrolyte Moving Boundary Systems

The solution of the moving boundary equation for the general case of n ions is difficult indeed with any assumption as to the concentration dependence of the relative mobility other than that this mobility is constant. Since this assumption is approximately valid for dilute solutions of completely dissociated electrolytes Dole's theory has come to be known as the strong-electrolyte moving boundary theory. Proteins are, however, not strong electrolytes. The success of the theory in accounting for the free electrophoresis of proteins resulted in large part from the fact that the composition of the medium with respect to small ions and molecules was such that the pH and ionic strength were approximately constant throughout the boundary system and these are the important variables in determining the mobility of a protein ion. Even so, it has been noted that correction for the pH changes across the boundaries has improved agreement between theory and observation.

Many systems are encountered, however, in which the departure of the patterns from enantiography exceeds that for which the strong-electrolyte theory can account. This is especially true of systems in which protein-protein interaction occurs and of many proteins outside of the isoelectric region. In these cases progress has been made with the aid of the concepts of the ion constituent and the ion constituent mobility. The weak-electrolyte moving boundary theory utilizing these concepts has been developed by Svensson (29) and by Alberty and associates (30–33).

A. The Ion Constituent

In the case of weak and incompletely dissociated electrolytes the kinetic units of interest, i.e. those capable of systematic motion relative to the solvent, are not only the simplest charged units into which the solute dissociates but also the complexes resulting from the association of these units. The classic example is cadmium iodide. In his work on the aqueous solutions of this salt Hittorf observed (2) that the transference number of the cadmium decreased from a value of 0.444 at 0.01 C to -0.120 at 1 C. The value at the lower concentration is that to be expected from the measured conductance and Kohlrausch's law of independent ion mobilities if the equilibrium

$$CdI_2 = Cd^{++} + 2I^-$$

is the only one to be considered and is displaced to the right. The negative value for T_{Cd} at 1 C means, however, that with cadmium electrodes more CdI_2 has accumulated, on electrolysis, at the anode than that produced by the electrode process. This is explained by assuming the existence, in solution, of the complexes CdI^+, CdI_2, CdI_3^-, CdI_4^{--}, etc., in addition to the simple Cd^{++}. Although transference and moving boundary experiments can seldom distinguish between these various complexes, such measurements are of value in suggesting their existence and in providing some of the data required for their characterization, as shown by Alberty and King (34).

With the preceding example in mind, the ion constituent may be defined as an ion-forming portion of an electrolyte including that actually present as ions and that potentially present in complexes with other ions including neutral molecules. As in the selection of Gibbs' components for application of the phase rule some discretion is left to the investigator in the choice of the ion constituents. At pH 7 and below, the simplest phosphate ion species that needs to be considered is HPO_4^{--} whereas in the alkaline range it would be PO_4^{---}. On the other hand recognition must be given to the fact that some solutes, e.g. NH_3 and CO_2 are not electrolytes, i.e. ionogenic, until they have reacted with the solvent water.

Although the charge on an ion is an integral multiple of that on the electron or proton the time average of this charge can, in the case of the ion constituents of weak electrolytes, have fractional values. Owing to the rapidity with which most ionic equilibria are established an ion constituent appears to migrate as a single species. In a homogeneous solution these equilibria are not disturbed by the passage of the current, but this is not the case at a boundary where the tendency of the various complexes to become separated necessitates readjustments of the equilibria. It is often possible to choose the end solutions so that an ion constituent disappears across a boundary. The boundary then sweeps out of the region through which it moves all of the given ion constituent, whether that constituent is or is not present as free ions.

B. Transport of Ion Constituents

A complex has the dual role of transporting charge and constituent, and the ratio of charge to constituent is a ratio of integers that generally varies from one complex to another. The iodide ion constituent of aqueous cadmium iodide affords an example in which the constituent is present, to different degrees of association, in cationic, neutral, and anionic complexes. A special case of importance (31) is the anion constituent of a polybasic acid, H_nR, that dissociates progressively to R^{n-} and is taken as disappearing from the α solution across the $\alpha\beta$ boundary of Fig. 5. The concentrations, m_j, of the complexes, $H_nR \ldots R^{n-}$, will be expressed in gram formula weights per milliliter and a complex is identified by the number, j, of electronic charges on it. The Faraday equivalents carried by the j complex through the plane b of Fig. 5 is jm_ju_j/κ^α per coulomb of electricity passed, and by all complexes it is $(1/\kappa^\alpha)\sum_{j=1}^{n} jm_ju_j$. Here the summation is from 1 to n since the mobility u_0 of the undissociated weak acid is taken as zero. The gram formula weight of constituent thus transported is $(1/\kappa^\alpha)\sum_{1}^{n} m_ju_j$ and must be equal, if the α solution is to remain homogeneous, to the formula weight of constituent, both ionized and uncharged, in the volume $v^{\alpha\beta}$ through which the boundary moves, i.e.

$$\frac{1}{\kappa^\alpha}\sum_{1}^{n} m_ju_j = v^{\alpha\beta}\sum_{0}^{n} m_j = v^{\alpha\beta}\overline{m}_R$$

Here $\overline{m}_R = \sum_{0}^{n} m_j$ is the concentration of the constituent in all complexes including the uncharged one for which $j = 0$. Thus the constituent mobility $\bar{u}_{\mathrm{R}}^{\alpha}$ computed from the boundary displacement as in the case of strong electrolytes, equation (18), is

$$\bar{u}_{\mathrm{R}}^{\alpha} = v^{\alpha\beta}\kappa^{\alpha} = (1/\bar{m}_{\mathrm{R}}^{\alpha}) \sum_{1}^{n} m_j^{\alpha} u_j^{\alpha} \tag{31}$$

In the boundary zone the uncharged and less mobile complexes must dissociate into more mobile forms if the constituent is to disappear across the boundary. Moreover, if the boundary does not remain sharp as it moves and the first moment of the refractive index gradient curve is taken as the boundary position, the centroids of any constituents whose concentration may change through the boundary must remain congruent for equation (31) to be valid. The boundary considered here is thus a special case of a reaction boundary in that the very mobile hydrogen ion of low equivalent weight is one of the reactants.

Since the fraction, α_j, of the constituent present as the j complex is $m_j^{\alpha}/\sum_{0}^{n} m_j^{\alpha}$, equation (31) is equivalent to the one suggested by Tiselius (6), i.e. $\bar{u} = \sum_{0}^{n} \alpha_j u_j$. If the R constituent is also present in the β solution (Fig. 5) and the boundary moves, on passage of the current, without forming a new homogeneous phase or separation of centroids the same considerations that led to equation (6) for strong electrolytes gives

$$\frac{\bar{u}_{\mathrm{R}}^{\alpha}\bar{m}_{\mathrm{R}}^{\alpha}}{\kappa^{\alpha}} - \frac{\bar{u}_{\mathrm{R}}^{\beta}\bar{m}_{\mathrm{R}}^{\beta}}{\kappa^{\beta}} = v^{\alpha\beta}(\bar{m}_{\mathrm{R}}^{\alpha} - \bar{m}_{\mathrm{R}}^{\beta}) \tag{32}$$

where the bars denote constituent mobilities and concentrations. Since this relation is homogeneous in the concentrations, these may also be taken as formula weights per liter. As with strong electrolytes m_j is a signed concentration and m_0 is taken with the sign of the constituent.

By further restricting the weak electrolytes to monovalent weak acids of the type HR, $\bar{u}_{\mathrm{R}}\bar{m}_{\mathrm{R}} = u_{\mathrm{R}}m_{\mathrm{R}}$ from equation (31), and equation (32) becomes

$$\frac{u_{\mathrm{R}}^{\alpha}m_{\mathrm{R}}^{\alpha}}{\kappa^{\alpha}} - \frac{u_{\mathrm{R}}^{\beta}m_{\mathrm{R}}^{\beta}}{\kappa^{\beta}} = v^{\alpha\beta}(\bar{m}_{\mathrm{R}}^{\alpha} - \bar{m}_{\mathrm{R}}^{\beta}) \tag{33}$$

Defining relative mobilities and conductances as with strong electrolytes, and assuming the r's to be constant,

$$\frac{m_{\mathrm{R}}^{\alpha}}{\sigma^{\alpha}} - \frac{m_{\mathrm{R}}^{\beta}}{\sigma^{\beta}} = v^{\alpha\beta}\left(\frac{\bar{m}_{\mathrm{R}}^{\alpha}}{r_{\mathrm{R}}} - \frac{\bar{m}_{\mathrm{R}}^{\beta}}{r_{\mathrm{R}}}\right)$$

Summing over all constituents, except hydrogen and hydroxyl, whose conductances are assumed to be negligible, and recalling the condition for electrical neutrality,

$$v^{\alpha\beta}\left(\Sigma\,\frac{\bar{m}_{\mathrm{R}}{}^{\alpha}}{r_{\mathrm{R}}} - \Sigma\,\frac{\bar{m}_{\mathrm{R}}{}^{\beta}}{r_{\mathrm{R}}}\right) = 0$$

If $v^{\alpha\beta} \neq 0$

$$\Sigma\,\frac{\bar{m}_{\mathrm{R}}{}^{\alpha}}{r_{\mathrm{R}}} = \Sigma\,\frac{\bar{m}_{\mathrm{R}}{}^{\beta}}{r_{\mathrm{R}}} = \bar{\omega} \qquad (34)$$

which will be recognized as the regulating function with the constituent concentration replacing the ion concentration.

Alberty (31) has ascertained the conditions for a boundary, across which two weak electrolyte constituents of the uncharged monovalent acid type disappear in opposite directions, to move as a steady state. In the system AR, HR (γ) ← AS, HS (β) :: AS, HS (α), if

$$|\bar{u}_{\mathrm{R}}{}^{\beta}E^{\beta}| > |\bar{u}_{\mathrm{R}}{}^{\gamma}E^{\gamma}|,$$

where $\bar{u}_{\mathrm{R}}{}^{\beta}$ is the constituent mobility of a trace of R that may diffuse back into the homogeneous β solution, the $\beta\gamma$ boundary moves as a steady state. The composition of the β solution is computed with the aid of equation (33) and the assumption that the reaction in the boundary, $\mathrm{HR} + \mathrm{S}^- \rightarrow \mathrm{HS} + \mathrm{R}^-$, goes to completion so that $C_{\mathrm{HS}}{}^{\beta} = C_{\mathrm{HR}}{}^{\gamma}$. This assumption is a special case of the more general one that is made in work with weak acids and bases, namely, that the total concentration of weak electrolyte remains unchanged at each level in the channel. For most values of the acid:salt ratio, the $\beta\gamma$ boundary moves as a steady state if HR is the stronger acid and R has the higher absolute mobility. Acetate ← cacodylate is such a boundary and was observed by Nichol (35) to move as predicted.

The ion constituents of water, H^+, and OH^-, are unique in that it is not possible for either of these constituents to disappear across a boundary in aqueous media. For example, the boundary moving into aqueous sodium acetate with acetic acid behind (36) is very sharp indeed since the mobility of the hydrogen ion constituent in the acid is much less than that of the sodium ion and is still less in the relatively high concentration of acetate ion ahead of the boundary. The mobility of the hydrogen ion constituent is, however, not zero ahead of the boundary, and thus only the sodium ion disappears across this boundary. Valid transference measurements of the hydrogen ion in strong acids are made, however, by treating the constituent mobility of the hydrogen ion in the salt solution behind the boundary as part of the solvent correction.

V. Protein-Ion Interaction

A. *Neutral Salt Effects*

Although the pH and ionic strength are the important parameters in determining the mobility of a protein, it is frequently observed that substitution, at constant pH and ionic strength, of a portion of the buffer salt AR with a neutral salt AS produces a significant change, Δu, in the mobility. This suggests that the protein complexes with ions other than hydrogen are different in the two media and that the mobility given by $v^{\alpha\beta}\kappa^{\alpha}$ in the following system

$$\mathrm{A,R,S,P}(\alpha) \leftarrow \mathrm{A,R,S}(\beta) :: \mathrm{A,R,S}(\gamma) \tag{I}$$

is that, $\bar{u}_P$, of the protein ion constituent. The corresponding system for a cationic protein need not be given and R may not be more mobile than S.

It is difficult, however, to interpret the mobility change owing to the multicomponent nature of the medium. Even if the friction coefficient of the protein remains unaltered and the change in net charge Δz is taken as $\Delta\bar{u}/(d\bar{u}/dh)$ this is usually appreciably less than the number of S ions that are bound as determined by the analytical methods (37). Here $d\bar{u}/dh$ is the proportionality between mobility and net charge as determined from titration increments at the same ionic strength. The discrepancy arises from the competitive nature of the binding and the effect of bound salt ions on the protein-hydrogen ion equilibria. Thus the binding of S ions may be accompanied by changes in the numbers of A,R and hydrogen ions that are bound. Merely to write the formula of one of the many possible protein ion complexes as $PH_iA_jR_kS_l$ suggests the large number of equilibria involved. Evidence is also accumulating that the uncharged buffer acid, HR, frequently participates in the protein complexes.

Additional information regarding ion binding is obtained, however, with the aid of boundaries across which a small ion constituent disappears, in the presence of protein, from an end solution of known composition. In their study of the binding of methyl orange, S, by bovine serum albumin, P, Smith and Briggs (38) determined $\bar{u}_P$ in one series of experiments with the system I and $\bar{u}_S = v^{\alpha\beta}\kappa^{\alpha}$ in a second system, II, by omitting the dye from an end solution

$$\mathrm{A,R,S,P}(\alpha) \leftarrow \mathrm{A,R,P}(\beta) \leftarrow \mathrm{AR}(\gamma) :: \mathrm{AR}(\delta) \tag{II}$$

B. Moving Concentration Boundaries

In substituting a neutral salt for a portion of the buffer salt at least one new ion species is thereby introduced into the system and the Dole theory requires an additional moving boundary for each new species. Moreover, in (I) above the added ion is present in both end phases and the new boundary is a moving one across which no ion species disappears. Such a boundary is called a moving concentration boundary although the term false boundary is sometimes used (39). If, however, the protein solution is prepared by dialysis the concentration changes across the new boundaries are usually so small as to render them undetectable, a conclusion that can be confirmed by computations with the Dole theory. In the systems (I) above and (IV) and (V) below, the moving concentration boundaries have been omitted. On the other hand, if a second moving boundary is observed a comparison of its velocity, and the refractive index difference across it, with the computed values will frequently help to decide whether it is a moving concentration boundary or arises otherwise. The occasional occurrence, in serum and plasma patterns at pH 8.6, of a faint boundary migrating ahead of the albumin has occasioned speculation as to its origin (12, 39–41).

C. Protein-buffered Systems

A preferred, though more difficult, procedure for the study of small-ion binding is to dispense with the buffer electrolytes and utilize the buffer capacity of the protein to control the pH. In this manner Alberty and Marvin (42) have studied the binding of chloride ion by bovine serum albumin, P, with the aid of the following systems

$$\mathrm{NaCl,P}(\alpha) \leftarrow \mathrm{NaCl}(\beta) :: \mathrm{NaCl}(\gamma) \tag{III}$$

$$\mathrm{LiCl,P}(\gamma) :: \mathrm{LiCl,P}(\beta) \rightarrow \mathrm{NaCl,P}(\alpha) \tag{IV}$$

$$\mathrm{NaCl,P}(c) \leftarrow \mathrm{NaClO_3,P}(b) :: \mathrm{NaClO_3,P}(a) \tag{V}$$

Since the end solutions containing NaCl and protein are identical, the constituent mobilities $\bar{u}_P$, $\bar{u}_{\mathrm{Na}}$ and $\bar{u}_{\mathrm{Cl}}$, for that solution are obtained from the displacement of the boundary $\alpha\beta$ in III, $\alpha\beta$ in IV, and bc in V, respectively. By omission of the protein from IV and V, these systems may be used to obtain the mobilities, u_{Na} and u_{Cl}, of the free ions. If the protein is below its isoelectric pH the boundary in (III) moves with the current.

Of the many assumptions (43) that can be made regarding the nature of the small-ion binding, one of the simplest is that all of the protein is present as a single complex, PCl_i. Then

$$\bar{m}_{\mathrm{Cl}}\bar{u}_{\mathrm{Cl}} = m_{\mathrm{Cl}}u_{\mathrm{Cl}} + \mathrm{i}m_{\mathrm{PCl}_i}u_{\mathrm{PCl}_i}$$

and

$$\bar{m}_{\mathrm{P}}\bar{u}_{\mathrm{P}} = m_{\mathrm{PCl}_i}u_{\mathrm{PCl}_i}$$

so that

$$\bar{m}_{\mathrm{Cl}}\bar{u}_{\mathrm{Cl}} - m_{\mathrm{Cl}}u_{\mathrm{Cl}} = \mathrm{i}\bar{m}_{\mathrm{P}}\bar{u}_{\mathrm{P}} \tag{35}$$

Moreover

$$\bar{m}_{\mathrm{Cl}} = m_{\mathrm{Cl}} + \mathrm{i}m_{\mathrm{PCl}_i}$$

$$\bar{m}_{\mathrm{P}} = m_{\mathrm{PCl}_i}$$

so that

$$m_{\mathrm{Cl}} = \bar{m}_{\mathrm{Cl}} - \mathrm{i}\bar{m}_{\mathrm{P}} \tag{36}$$

Elimination of the concentration, m_{Cl}, of free chloride between equations

(35) and (36) gives

$$\mathrm{i} = \frac{\bar{m}_{\mathrm{Cl}}(\bar{u}_{\mathrm{Cl}} - u_{\mathrm{Cl}})}{\bar{m}_{\mathrm{P}}(\bar{u}_{\mathrm{P}} - u_{\mathrm{Cl}})}$$

The chloride ion mobility, u_{Cl}, is that of the free chloride in the protein solution whose viscosity is greater than that of the protein-free chloride solution of the same ionic strength actually used for the determination of u_{Cl}. If the assumptions are now made that the protein does not bind sodium and that the free sodium and chloride ion mobilities are reduced proportionally by the viscous effect, $u_{\mathrm{Cl}} = u^{\circ}_{\mathrm{Cl}}\bar{u}_{\mathrm{Na}}/u^{\circ}_{\mathrm{Na}}$, where the superscript indicates mobilities in protein-free solution.

$$\mathrm{i} = \frac{\bar{m}_{\mathrm{Cl}}(\bar{u}_{\mathrm{Cl}}u^{\circ}_{\mathrm{Na}} - u^{\circ}_{\mathrm{Cl}}\bar{u}_{\mathrm{Na}})}{\bar{m}_{\mathrm{P}}(\bar{u}_{\mathrm{P}}u^{\circ}_{\mathrm{Na}} - u^{\circ}_{\mathrm{Cl}}\bar{u}_{\mathrm{Na}})} \tag{37}$$

The results of Alberty and Marvin are assembled in Table IV and are in agreement with values of i obtained by other methods, the molecular weight of the protein being taken as 69,000.

VI. PROTEIN-PROTEIN INTERACTION

The term protein is used here in the sense of a macromolecular ion, whereas in the interaction considered above the ion was visualized as small. A practical distinction between protein-ion and protein-protein interaction is afforded by the permeability of the dialysis membrane to one, or neither, of the reactants. Moreover, protein-ion interaction is frequently described with the aid of the Langmuir adsorption isotherm, whereas a simple bimolecular association is often adequate for protein-protein interaction.

A. *Effect of Interaction on Electrophoretic Patterns*

An early, but fairly typical, electrophoretic study of protein interaction involved mixtures of ovalbumin P and yeast nucleic acid N (45). In a 0.1 (sodium acetate buffer at pH 5.34 and above the patterns, Fig. 8, are those of a normal mixture, whereas at 4.63, a pH slightly above that at which partial precipitation occurs, the patterns, Fig. 9, exhibit departures from enantiography that cannot be explained by the Dole theory. At this pH the protein still has a small negative mobility, -0.30×10^{-5}, corresponding to the displacement of the $\beta\gamma$ boundary, but is not sufficiently mobile to permit resolution of this boundary and the buffer concentration boundary $\gamma\delta$. On the other hand the conjugate rising boundary *bc* corresponds to a mobility 8 times that of $\beta\gamma$. Similarly the displacement of the *cd* boundary corresponds to the mobility of free nucleic acid whereas that of the $\alpha\beta$ boundary is appreciably smaller. Although less apparent the areas of the *bc* and $\alpha\beta$ boundaries exceed the values they would have if no inter-

TABLE IV

Binding of Chloride by Bovine Serum Albumin in 0.15 *M* Sodium Chloride at 0°C.

pH	—	7.00	7.00	7.00	7.00	5.40	3.20
Gm. albumin/100 ml.	0	2	4	6	8	4	4
$M_P \times 10^4$		−2.90	−5.80	−8.70	−11.60	−5.80	5.80
$\bar{u}_{Cl} \times 10^5$	−36.19	−34.40	−32.72	−31.10	−29.40	−32.62	−29.59
$\bar{u}_{Na} \times 10^5$	21.59	20.84	20.11	19.38	18.65	20.10	20.10
$\bar{u}_P \times 10^5$		−4.55	−4.41	−4.26	−4.11	−2.75	4.00
i [Eq. (37)]		$9._0$	$8._8$	$8._4$	$8._8$	$9._0$	$28._2$
i (human albumin) (44)				8		11	31

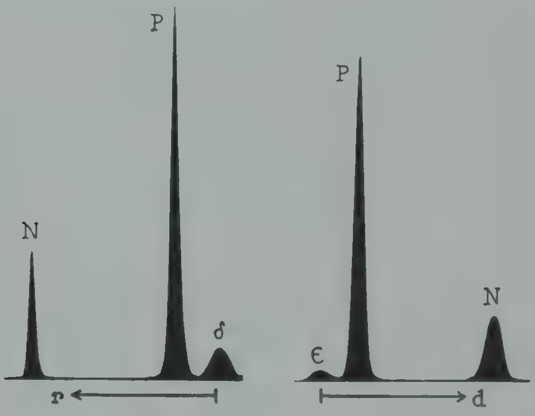
action occurred. Equally indicative of such interaction is the skewness of the $\alpha\beta$ boundary, a feature of the patterns that has been recognized only since the development, by Gilbert and Jenkins (46) of the concept of constituent mobility spreading to be considered later in this chapter.

If k_f and k_r are the velocity constants for the forward and reverse reactions

$$P + iN \underset{k_r}{\overset{k_f}{\rightleftarrows}} PN_i$$

the equilibrium constant is $K = k_r/k_f$ and the following possibilities arise.

1. If k_f is small and k_r large the complex is essentially completely dissociated and the pattern is that of a normal mixture of P and N (Fig. 8).



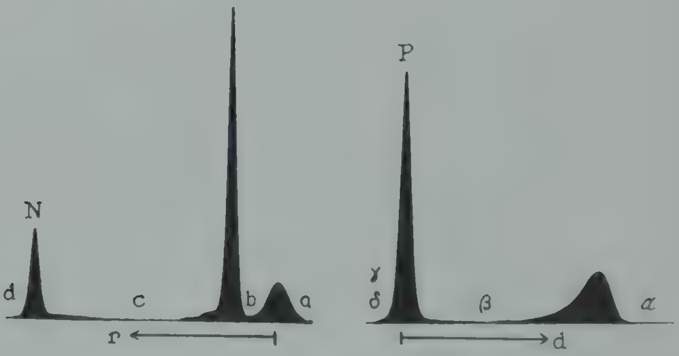


Fig. 8. Electrophoretic patterns of ovalbumin-yeast nucleic acid mixtures at pH 5.3.

Fig. 9. Electrophoretic patterns of ovalbumin-yeast nucleic acid mixtures at pH 4.6.

2. If k_f is large and k_r small then K is small and the behavior is that of a normal mixture of complex and either P or N, depending upon which is in excess. If neither is in excess, a single moving boundary due to the complex is present in each side of the channel (46a).

3. If k_f and k_r are both small and of similar magnitude, finite concentrations of P, N, and PN_i are present at equilibrium and the behavior is that of a noninteracting mixture of three components.

4. If the velocity constants are such that significant electrophoretic separation can occur in the boundaries during the "half-lives" of the interacting species the patterns would be expected to depend upon the rate of separation. This possibility has been considered for an isomerization reaction $P \rightleftharpoons P'$ (47) and for a polymerization or dissociation $iP \rightleftharpoons P_i$ (48, 49).

5. The case in which k_f and k_r are large and of similar magnitude appears to be the one most frequently encountered and affords, as shown below, an explanation of the P-N interaction at pH 4.63. The equilibria in the boundary layers are then adjusted as rapidly as required by the electrophoretic separation and not more than two moving boundaries are observed in each side of the channel.

B. Ovalbumin-Nucleic Acid Interaction at pH 4.63 and 0°C.

Since only the molecular weight, M_P, of the protein is known, the reaction is assumed to be simply $P + N \rightleftharpoons PN$. The two parameters to be evaluated are then the equilibrium constant K and the factor i by which the total concentration of N in grams per cent is multiplied to give a concentration that is comparable, on a molar scale, with that of P in grams per cent. The parameter i would thus be interpreted as the molecular weight ratio, M_P/M_N. In the present example i is selected to make K constant and has the value 2.0, leading to the value of about 20,000 for M_N. Although the interpretation of the constants is somewhat different, the reaction postulated here is case III of Alberty and Marvin (43).

The systems represented by Fig. 9 are

$$\mathrm{A,R,P,N,PN}(\alpha) \leftarrow \mathrm{A,R,P}(\beta) \leftarrow \mathrm{A,R}(\gamma) :: \mathrm{A,R}(\delta)$$

and $$\mathrm{A,R}(d) \leftarrow \mathrm{A,R,N}(c) \leftarrow \mathrm{A,R,P,N,PN}(b) :: \mathrm{A,R,P,N,PN}(a)$$

where the N ion constituent disappears across the $\alpha\beta$ boundary and the P ion constituent across the bc boundary. Elimination of m_{PN}^{α} between the expressions for the constituent concentrations gives

$$\bar{m}_P^{\alpha} - m_P^{\alpha} = \bar{m}_N^{\alpha} - m_N^{\alpha} \tag{38}$$

whereas $m_{PN}^{\alpha}u_{PN}^{\alpha}$ may be similarly eliminated between the constituent mobilities to give

$$\bar{m}_P^{\alpha}\bar{u}_P^{\alpha} - m_P^{\alpha}u_P^{\alpha} = \bar{m}_N^{\alpha}\bar{u}_N^{\alpha} - m_N^{\alpha}u_N^{\alpha} \tag{39}$$

The concentration of free nucleic acid is obtained by elimination of m_P^{α} between the foregoing relations,

$$m_N{}^\alpha = \frac{\bar{m}_N{}^\alpha(\bar{u}_N{}^\alpha - u_P{}^\alpha) - \bar{m}_P{}^\alpha(\bar{u}_P{}^\alpha - u_P{}^\alpha)}{u_N{}^\alpha - u_P{}^\alpha}$$

If the electrophoresis were ideal except for the P-N interaction the conductances of all phases would be equal and $\bar{u}_N{}^\alpha$, $\bar{u}_P{}^\alpha$, $u_N{}^\alpha$ and $u_P{}^\alpha$ could be identified with $v^{\alpha\beta}$, v^{bc}, v^{cd} and $v^{\beta\gamma}$, respectively. Thus m_N and hence

$$K = \frac{m_N m_P}{m_{PN}} = \frac{m_N(\bar{m}_P - \bar{m}_N + m_N)}{\bar{m}_N - m_N} \tag{40}$$

could be evaluated without measurement of pattern areas. However, *ab* is not a concentration boundary as in the case of strong electrolytes since all ions cannot remain in the same relative proportions across the boundary and preserve the P-N equilibrium. Smith and Briggs (38) first recognized this feature of the *ab* boundary. If *ab* is assumed to be a concentration boundary correction of $\bar{u}_P{}^b$ and $u_N{}^c$ for the dilution can be made but the resulting values of K are not as constant as those obtained from the descending boundaries as follows.

The concentration of protein in the β solution can be obtained from the area of the $\beta\gamma$ peak and also appears in the moving boundary equation, equation (32), for this constituent

$$\frac{\bar{m}_P{}^\alpha \bar{u}_P{}^\alpha}{\kappa^\alpha} - \frac{m_P{}^\beta u_P{}^\beta}{\kappa^\beta} = v^{\alpha\beta}(\bar{m}_P{}^\alpha - m_P{}^\beta) \tag{41}$$

Assuming $\kappa^\alpha = \kappa^\beta$, noting that $v^{\alpha\beta}\kappa^\alpha = \bar{u}_N{}^\alpha$ and eliminating $\bar{m}_P{}^\alpha \bar{u}_P{}^\alpha$ and $m_P{}^\alpha$ between equations (38), (39), and (41) gives

$$m_N{}^\alpha = (\bar{m}_N{}^\alpha + m_P{}^\beta - \bar{m}_P{}^\alpha)\,\frac{\bar{u}_N{}^\alpha - u_P{}^\alpha}{u_N{}^\alpha - u_P{}^\alpha} \tag{42}$$

In the computation of K, with the aid of equations (42) and (40), that are summarized in Table V, $u_P{}^\alpha = -0.30 \times 10^{-5}$ and $u_N{}^\alpha = -12.0 \times 10^{-5}$ are the mobilities of P and N in 1% solutions of the pure constituents.

TABLE V

THE EQUILIBRIUM CONSTANT AT pH 4.63, $\Gamma/2 = 0.1$ AND 0°C. FOR THE ASSOCIATION OF OVALBUMIN (P) AND YEAST NUCLEIC ACID (N) FROM ELECTROPHORETIC PATTERNS:
$M_P/M_N = i = 2.0$ $-u_P = 0.30 \times 10^{-5}$ $-u_N = 12.0 \times 10^{-5}$

$p_N(\sim m_N{}^\alpha/i)$ (gm./100 ml.)	$p_P(\sim m_P{}^\alpha)$	$p_P{}^\beta$	$\bar{u}_N{}^\alpha \times 10^5$	K (gm./100 ml.)
0.63	0.60	0.502	10.46	1.39
0.26	1.13	1.031	8.49	1.18
0.51	1.13	0.960	9.18_5	1.30
0.66	1.17	0.964	9.43	1.38
1.11	1.19	0.872	9.89_5	1.25

VII. Reaction Boundaries

A. Constituent Mobility Spreading

In the case of protein-protein interaction Gilbert (50, 51) and Gilbert and Jenkins (46) have considered the effect of re-equilibration in the boundary layers upon the spreading of the boundary. An example is afforded by a protein that is present in solution as a dimer, B_2, in rapidly reversible equilibrium with monomer B where $u_{B_2} > u_B$. At the descending boundary across which this constituent disappears, the proportion of monomer in the dilute trailing layers of the boundary is greater than in the concentrated leading layers. The constituent mobility thus decreases with increasing dilution, and the boundary spreads as it descends even in the absence of diffusion. Such a boundary will be termed a reaction boundary.

If n is greater than 2 in the equilibrium $nB \rightleftharpoons B_n$ the refractive index gradient curve may be bimodal, i.e. have two maxima (51). Bimodality can also arise if the equilibrium $B + C \rightleftharpoons BC$ is being adjusted in the boundary (46). In the case of the ovalbumin-nucleic acid mixtures (Fig. 9) the interaction was not strong enough to produce bimodality but did cause the leading, descending boundary at pH 4.63 to be skew. The interaction of ovomucoid and yeast nucleic acid (45) is such, however, as to give rise to bimodal reaction boundaries. These are indicated by the brackets in Fig. 10.

An essential characteristic of a moving boundary is that it separates two homogeneous phases. Consequently a bimodal reaction boundary should be considered a single boundary since a homogeneous phase cannot be generated between the two maxima. It will be noted that the gradient does not become zero at any level in the bracketed boundaries of Fig. 10.

The concept of the reaction boundary with rapid re-equilibration thus affords an explanation for many patterns that have long puzzled investigators. Although the theory is still being developed, the following reactions are considered in the order listed: (a) the polymerization $nB \rightleftharpoons B_n$, or dissociation into identical fragments, (b) the bimolecular association $B + C \rightleftharpoons BC$ and (c) the isomerization $B \rightleftharpoons B'$.

B. The Polymerization Reaction

In the polymerization reaction

$$nB \rightleftharpoons B_n$$

the concentrations of monomer B (M) and polymer B_n (P) will be expressed in grams per unit volume and will be designated *m* and *p*, respectively. If the boundary across which this constituent disappears starts

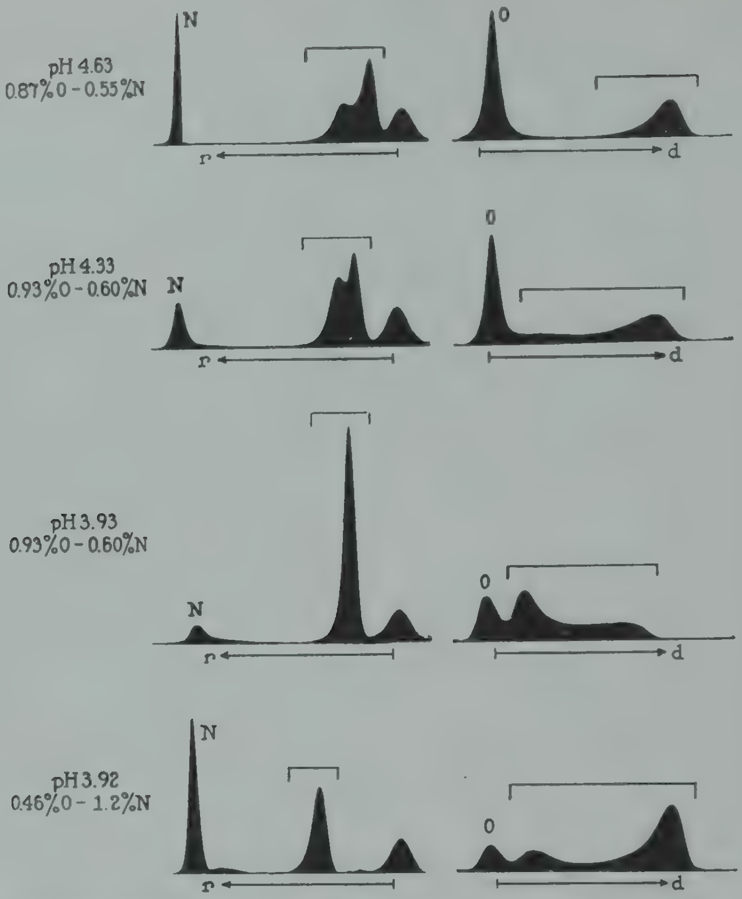


FIG. 10. Electrophoretic patterns of ovomucoid-yeast nucleic acid mixtures in 0.1 N NaAc buffers.

as a sharp one at $x = t = 0$ and moves into the α solution in the direction of increasing x it would thus correspond to the descending boundary. Even if ideal electrophoresis is assumed, except for the reaction in the boundary, and diffusion is neglected, this boundary exhibits constituent spreading if $u_P > u_M$. In a thin layer of solution in the boundary both m and p increase with x and in the absence of interaction the conservation relations would be $\partial m/\partial t = -v_M \partial m/\partial x$ and $\partial p/\partial t = -v_P \partial p/\partial x$ where the velocity, v, is Eu. With rapid re-equilibration the relation is

$$\frac{\partial m}{\partial t} + v_M \frac{\partial m}{\partial x} + \frac{\partial p}{\partial t} + v_P \frac{\partial p}{\partial x} = 0$$

Moreover

$$Kp = m^n$$

Elimination of p between these expressions gives

$$\frac{dx}{dt}\left(1 + \frac{nm^{n-1}}{K}\right) = v_M + v_P \frac{nm^{n-1}}{K} \qquad (43)$$

where $dx/dt = -(\partial m/\partial t)/(\partial m/\partial x)$ and may be interpreted as the velocity with which a layer of constant m in the boundary moves. Equation (43) may be integrated to give

$$\frac{\mathrm{n}m^{\mathrm{n}-1}}{K} = \frac{(x/t) - \mathrm{v_M}}{\mathrm{v_P} - (x/t)} \tag{44}$$

In the boundary m increases with x from 0 to m_0, its value in the body of the solution, whereas the right side of equation (44) varies from 0 at $x/t = \mathrm{v_M}$ to ∞ as x/t approaches $\mathrm{v_P}$. Thus the trailing edge of the boundary has the velocity of pure monomer whereas the velocity of the leading edge depends upon the constituent concentration $\bar{m}_0 = m_0 + p_0$ and K but is always less than $\mathrm{v_P}$. In the coordinate system used by Gilbert (51) the polymer in the boundary zone is considered as moving with respect to stationary monomer. The coordinate transformation is

$$\delta = \frac{(x/t) - \mathrm{v_M}}{\mathrm{v_P} - \mathrm{v_M}}$$

and equation (44) becomes

$$\frac{\mathrm{n}m^{\mathrm{n}-1}}{K} = \frac{\delta}{1 - \delta}$$

where the positive character of m restricts δ to $0 < \delta < 1$. The constituent concentration at any level in the boundary is

$$\bar{m} = m + p = \left(\frac{K}{\mathrm{n}}\frac{\delta}{1-\delta}\right)^{\frac{1}{\mathrm{n}-1}}\left(1 + \frac{1}{\mathrm{n}}\frac{\delta}{1-\delta}\right)$$

Setting the second derivative of $\bar{m}$ with respect to x equal to zero indicates that for values of $\mathrm{n} > 2$ the gradient curve is bimodal with the minimum at $\delta = (\mathrm{n} - 2)/(3\mathrm{n} - 3)$.

In the other side of the channel the dissociation of polymer in the dilute leading edges of the rising boundary partially compensates for the spreading effect of diffusion and this boundary remains sharp.

C. The Bimolecular Association Reaction

Boundaries in which the reaction $\mathrm{B} + \mathrm{C} \rightleftharpoons \mathrm{BC}$ is re-equilibrated are of considerable interest in electrophoresis. The conservation equations

$$\frac{\partial b}{\partial t} + \mathrm{v_B}\frac{\partial b}{\partial x} = \frac{\partial c}{\partial t} + \mathrm{v_C}\frac{\partial c}{\partial x} = -\frac{\partial(bc)}{\partial t} - \mathrm{v_{BC}}\frac{\partial(bc)}{\partial x}$$

have been solved by Gilbert and Jenkins (46) and applied to a model in which $b_0 = 2$, $c_0 = 1$, $(bc)_0 = 1$, $\mathrm{v_B} = 10$, $\mathrm{v_C} = 8$ and $\mathrm{v_{BC}} = 14$. In the body of this solution, α, the velocity of the B constituent, $11\frac{1}{3}$, is greater than that, 11, of the C constituent and a solution, β, of pure C should form behind the descending reaction boundary $\alpha\beta$.

$$\mathrm{A,C,R}(\beta) \rightarrow \mathrm{A,B,C,BC,R}(\alpha)$$

The weak-electrolyte moving boundary, equation (32), gives 0.20 as the concentration of C in the β solution, whereas 0.26 is obtained when account of the reaction in the boundary is taken. In addition to being bimodal this $\alpha\beta$ boundary thus illustrates another significant property of reaction boundaries. If the concentration of more than one constituent changes in a reaction boundary their centroids generally separate as the boundary spreads.

The assumption of ideal electrophoresis except for the boundary reaction implies sufficient concentrations of buffer electrolytes to suppress pH and conductivity changes in the system. Many of the boundaries encountered in the electrophoresis of proteins in acidic media (52–56) appear to be bimodal reaction boundaries. Here, however, it has been suggested (54, 56) that the reaction is between the protein and the uncharged weak buffer acid or base. The theory for this interaction has not yet been developed.

D. The Isomerization Reaction

In the isomerization reaction

$$B \underset{k_r}{\overset{k_f}{\rightleftarrows}} B'$$

the concentrations of the isomers B and B′ are independent of the constituent concentration at equilibrium. Thus with rapid re-equilibration the constituent mobility does not vary through a boundary across which an isomerizing constituent disappears and spreading from this source does not occur. Such a boundary is of interest, however, since here the effect of *slow* re-equilibration may be investigated uncomplicated by constituent spreading.

Some of the properties of a boundary in which a slowly reversible isomerization occurs may be visualized with the aid of Fig. 11 for the case in which $k_f = k_r$ and $u_B = 2u_{B'}$. In this figure the ideal electrophoresis of the isomers B and B′ has been depicted as occurring in steps such that during a short time interval, $\Delta t \simeq 1/k$, no readjustment of the equilibrium in the boundary occurs, followed by instantaneous adjustment prior to the next interval. At zero time the boundaries in the two sides of the channel are at x_1 and x_2 (Fig. 11, a), and the full line of this figure could thus represent the variation of concentration of either B or B′ with distance. In Δt seconds the B isomer moves two increments of distance to the right, the full line of Fig. 11, b, whereas the B′ isomer migrates half this distance, the dashed line. Instantaneous equilibration then gives the full line of Fig. 11, b′ as representing the concentration distribution of both B and B′. In the next time interval the distribution of material just prior to equilibra-

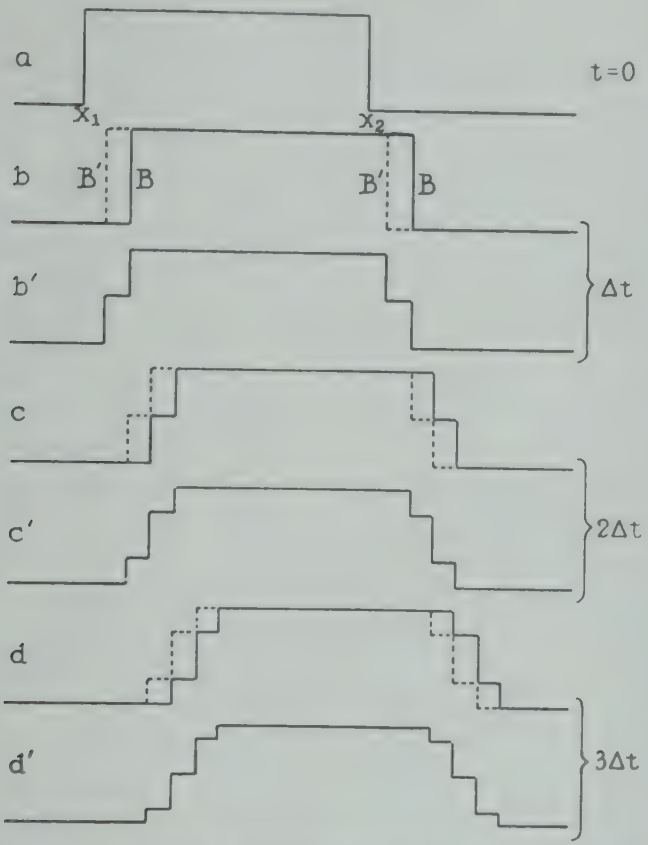


FIG. 11. Irreversible boundary spreading due to slow isomerization.

tion is shown in Fig. 11, c and after equilibration in Fig. 11, c′. Figs. 11, d and d′ represent the sequence of events in the third interval.

Since the heights of successive steps in the figure are proportional to the binomial coefficients, the gradient curve is a symmetrical one, spreading progressively with time and thus differing from spreading by diffusion, which is proportional to the square root of the time. It also differs from that due to protein heterogeneity since spreading continues on reversal of the current. Moreover, since enantiography of the conjugate patterns is preserved, it should also be possible to distinguish spreading due to slow isomeric re-equilibration from that due to field, pH, and constituent mobility effects. A construction similar to that of Fig. 11 for $k_f \neq k_r$ gives skew boundaries. In the theory developed by Cann and associates (47) diffusion has also been taken into account and cases considered in which bimodal boundaries occur. In all instances, however, the criteria suggested above should make it possible to recognize a boundary in which slow re-equilibration of isomers occurs. Field and Ogston (49) have treated a sedimenting boundary in which slow re-equilibration of monomer-polymer was assumed but found, on comparison with experiment, the skewing to be insensitive to the velocity constants. The assumption of rapid re-equilibration in the reaction boundaries encountered both in free electrophoresis and sedimentation appears to be valid in nearly all instances.

VIII. Reversible Boundary Spreading

A. *Multimodal and Unimodal Heterogeneity*

Since free electrophoresis affords a precise measure of one property of a protein ion, the mobility, it is frequently used to obtain evidence as to the purity of a protein preparation. In the case of human plasma in 0.1 C sodium diethylbarbiturate at pH 8.6 the mobilities are clustered (27) about values of -5.9, -5.1, -4.1, -2.8, -2.1, and -1.0×10^{-5} cm^2/volt-seconds and the corresponding maxima in the refractive index gradient curve are ascribed to albumin, α_1-, α_2-, β-globulin, fibrinogen, and γ-globulin, respectively. Such a protein mixture may be described as multimodally heterogeneous in that it may be separated, by preparative electrophoresis or other methods, into fractions such that the mobilities will be clustered about one of the above values when re-examined in the same buffer solvent. This represents a measure of purification. Frequently, however, when such a purified protein is examined at another pH or ionic strength, the mobilities are again found to cluster about more than one value. Thus crystalline ovalbumin migrates as a "single" protein at pH 3.9 (Fig. 7, a) but is bimodally heterogeneous at pH 5.34 (Fig. 7, b). Here Perlmann (57) has shown that the two boundaries are due to the presence of two phosphate groups in the major component and one such group in the minor component.

Furthermore, many preparations that migrate as a single protein throughout the height of the Tiselius cell split into two or more moving boundaries on prolonged electrophoresis with counterflow. This apparent heterogeneity must be interpreted as such with caution, however, since the splitting is usually restricted to the rising boundary and evidence for heterogeneity is most convincing if enantiography is preserved. Moreover, in countercurrent electrophoresis there is a "drainage error" in the flow of liquid in the channel (58), the effect of which can be ascertained by noting any decrease with time in the refractive index difference across the boundaries.

The failure of a protein to develop more than one moving boundary in a given buffer solvent does not imply that all of the protein ions have identical mobilities. A single protein ion in solution may be visualized as a dynamic system in rapidly shifting equilibria with the small ions and molecules of the medium and undergoing more or less continuous changes of shape, e.g. partial unfolding and refolding of the globular configuration and even partial uncoiling and recoiling of the α helix. Since these changes involve the breaking and reforming of hydrogen and other weak bonds, many of the resulting configurations have similar energies and thus are present in significant amounts in a solution of the protein. Since the mobility of a protein ion depends both on the net charge and the friction coefficient or shape this property also fluctuates with time about a mean value. Owing, however, to the rapidly reversible nature of the equilibria these mobility fluctuations are not detectable and this "reversible heterogeneity" probably cannot account for the reversible boundary spreading actually observed.

Reversible spreading is shown by most, if not all, proteins and differs from the types considered thus far in that the conjugate patterns remain enantiographic during electrophoresis while the boundary spreads more rapidly than from diffusion, whereas on reversal of the current the spreading is less rapid than from this source. This is the behavior that would result if the time-average mobilities were distributed about a mean value and may be described as unimodal heterogeneity.

B. The Heterogeneity Constant

Reversible boundary spreading was first observed by Tiselius and Horsfall (59, 60) with the hemocyanins, and the theory has been developed by Sharp and co-workers (61), Alberty and associates (62–65), and Brown and Cann (66). An example of reversible spreading is afforded by human γ_2-globulin in a 0.1 C sodium cacodylate-sodium chloride buffer at the average isoelectric pH of this protein, 7.27. In such studies it is essential to work at the isoelectric pH in order to preserve enantiography by minimizing pH and conductivity spreading. In Fig. 12 the gradients in the boundary at which current is flowing initially from buffer to protein solution are indicated by the full lines and are centered on the abscissa at the times they were recorded after the passage of the constant current began. The dashed curves represent the computed distribution of gradients if diffusion alone caused the spreading and at 300 minutes the current was reversed. It will be noted that, after passage of current from protein to buffer for an equal period, the spreading due to heterogeneity has been reversed and the gradient curve is that due to diffusion alone.

In the case of γ_2-globulin the mobility distribution is essentially Gaussian and the apparent diffusion coefficient D^* that may be computed from the gradient curves prior to reversal of the current increases linearly with the time, i.e.

$$D^* = D + (E^2\mathrm{h}^2/2)t$$

Here D is the true diffusion coefficient, E the electric field strength and h, the heterogeneity constant, is the standard deviation of the mobility

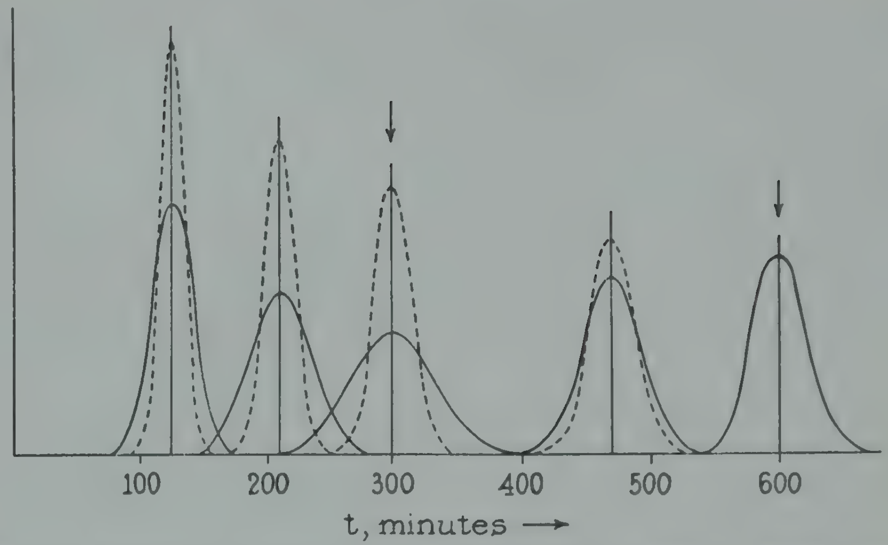


FIG. 12. Reversible boundary spreading of γ_2-globulin. (From Alberty (62)).

distribution. For γ_2-globulin at $\Gamma/2 = 0.1$ and 0°C., $h = 0.55 \times 10^{-5}$ cm²/volt-second (62). Since the standard deviation, i.e. the square root of the second moment, is one-half the length of the chord at the inflection points of a Gauss curve this value of h means that 68% of the protein ions have mobilities differing from zero by $\pm 0.55 \times 10^{-5}$ or less. The increased values of h observed at lower ionic strengths are satisfactorily explained by Henry's adaptation (67) of the Debye-Huckel theory to macromolecular ions.

If the spreading of a boundary that was sharp at $x = t = 0$ were due solely to unimodal heterogeneity, the refractive index gradient at a level x in the boundary would also represent the relative proportion of protein ions with the mobility x/Et. The relation

$$\mathrm{g}(u) = \mathrm{g}\left(\frac{x}{Et}\right) = \frac{Et}{n_1 - n_2}\frac{dn}{dx} \tag{45}$$

would then give the mobility distribution, $\mathrm{g}(u)$, where $n_1 - n_2$ is the difference in refractive index across the boundary. Since spreading by diffusion proceeds as the square root of the time whereas that due to heterogeneity is linear in this variable at constant field strength correction for diffusion can be made by extrapolating $\mathrm{g}(u)$, equation (45), to infinite time, i.e. zero reciprocal time. This is the procedure used by Baldwin *et al.* (65) in those instances in which the heterogeneity was not Gaussian. Gosting has shown (68) that after sufficient time the extrapolation becomes linear in $1/t$.

In the case of γ_2-globulin, where h is relatively large, there is little doubt that the heterogeneity arises, in part at least, from variations in the primary structure of the protein, i.e. from variations in the number, or variety, or sequence, or covalent crosslinking, or combinations thereof, of the amino acid residues in the peptide chain (69). In the case of crystalline proteins h is small, frequently less than the mobility increment per unit charge, but not zero. Here current research suggests that if variations in primary structure are present they are few in number and the question arises as to whether or not they are sufficient to account for the apparently continuous distribution of mobilities. Possibly if the theory of reaction boundaries can be extended to protein-small particle interaction, conditions will be found that preserve enantiography with this type of reversible spreading.

ACKNOWLEDGMENTS

The author is indebted to R. A. Alberty, V. P. Dole, and D. A. MacInnes for reviews of this chapter and to G. A. Gilbert for clarifying correspondence on reaction boundaries.

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Chapter 4

Moving Boundary Electrophoresis—Practice

L. G. LONGSWORTH

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I. Introduction

To study the migration of proteins and related materials in an electric field by the method of free electrophoresis it is necessary in the first place to form a sharp boundary between a solution of the material in a suitable medium—preferably a buffer, and the medium itself. In the case of a single protein, passage of the current then causes the boundary to move. For this movement to be a measure of the mobility of the protein it is desirable, first, that the electric field and pH in the neighborhood of the boundary be substantially constant; second, that the electrode processes do not involve the evolution of gas or other uncertain volume changes; third, that foreign ions do not penetrate into the regions in which the boundaries are moving; fourth, that the Joule heat generated by the passage of the current be dissipated without disturbance of the boundaries by convective circulation; and, finally, that provision be made for the observation and photography of the boundaries. As is well known, these conditions have been admirably met in the Tiselius apparatus (1), a description of which is the subject of this chapter.

II. The Tiselius Apparatus

A. *The Tiselius Cell*

A widely used form of the Tiselius cell is shown in perspective in Fig. 1 and consists of three sections that may be slid over each other along the planes *AA′* and *BB′*. The U-shaped channel provided by the cell is of rectangular cross section as shown in Fig. 1, b, which is a view from above of a horizontal plate. The height of the center section II is 92 mm. which, with horizontal plates of 3-mm. thickness, leaves 86 mm. clear height for observation of the boundaries. Since the separation of the two sides of

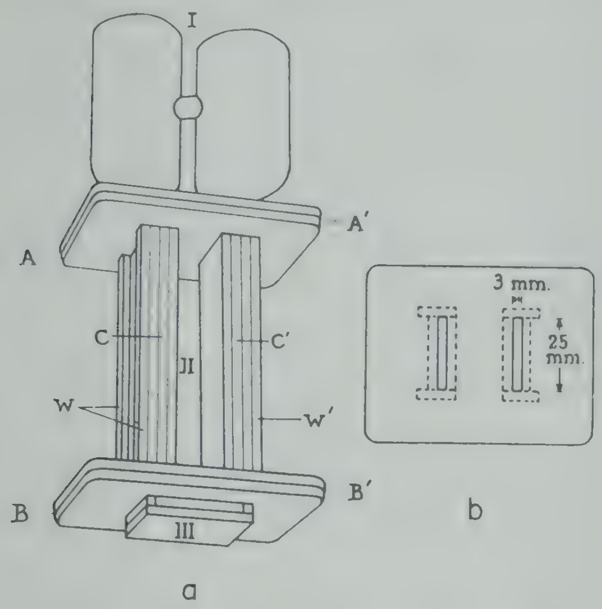

FIG. 1. The Tiselius electrophoresis cell.

the channel is 25 mm., and the channel cross section is 3 × 25 mm., about 11 ml. of protein solution are required to fill one side of the center section and the bottom section III. Consequently it is frequently referred to as the 11-ml. cell.

The channel width, $w = 3$ mm., is the important dimension in the control of convection; the depth, $a = 25$ mm., largely determines the optical sensitivity since light traverses the channel along this axis; while the resolving power for proteins whose mobilities differ only slightly depends on the clear height, 86 mm., unless recourse is had to countercurrent electrophoresis. In other cells one or more of the channel dimensions has been decreased in order to reduce the volume of protein solution required. The presently available cells (2, 3, 4, 5) range downward in channel width to 1 mm., in depth to 5 mm., and in height to 12 mm.

Except for the tubes of section I that connect with the electrode vessels shown below in Fig. 2, the cells are constructed of glass or quartz plates cemented together with an acid-resistant flux. The sliding motion along the planes AA' and BB' is used to obtain sharp boundaries initially between the protein and buffer solutions. With these interfaces lubricated with a mixture of two parts petroleum jelly plus one part mineral oil (1) and the sections aligned as shown in the figure, a continuous, leak-proof, U-shaped channel is formed. In view of the complexes that serum albumin and other proteins form with many fatty acids, a proprietary lubricant containing these materials should be used with caution as a substitute for the relatively inert one suggested above.

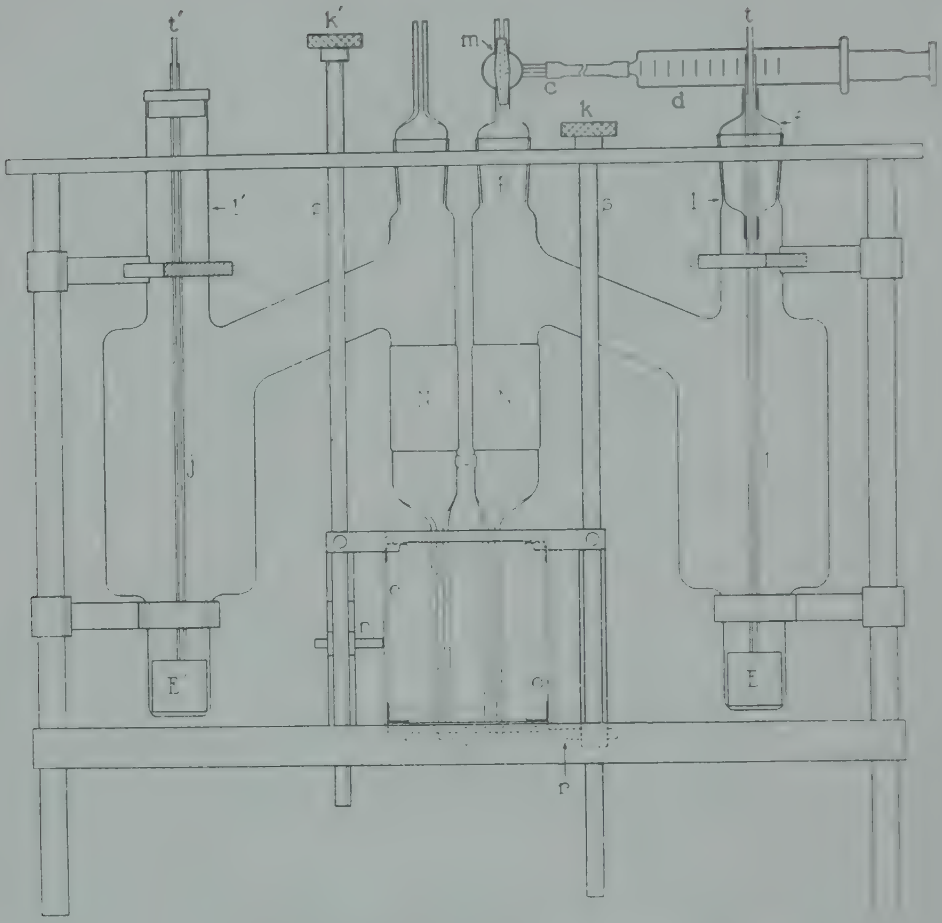

FIG. 2. Electrophoresis cell, electrode vessels, and support.

B. Manipulation of the Cell

A typical support for the cell and electrode vessels (6) is shown diagrammatically in Fig. 2, and three stages in the filling of the cell preparatory to electrophoresis in Fig. 3. With the assembled cell in position in the support, but without the electrode vessels connected, protein solution is introduced into the bottom section and this section displaced to the left, Fig. 3, a. The displacement is effected with the aid of the knob, *k* of Fig. 2, whose shaft carries an arm that presses against the edge of the horizontal plate of the bottom section. A similar knob *k′* permits shifts in the opposite direction and since the shafts *s* may be displaced vertically either the center or bottom sections may be thereby shifted.

Following the filling of the right side of section II with protein, and the rinsing and filling of the left side with buffer, the electrode vessels are connected as shown in Fig. 2, filled with buffer to the level of their side arms and the assembly placed in a thermostat regulating at about 1°C

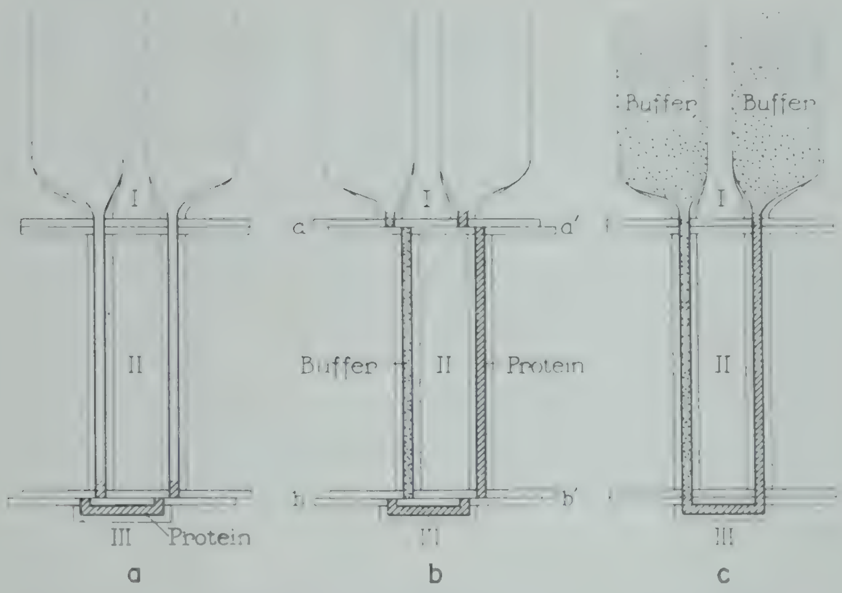

FIG. 3. Diagrams illustrating the initial formation of the boundaries.

After a brief interval for thermal equilibration, the center section is displaced to the right and the bottom section returned to the center position (Fig. 3, b). The excess protein solution is then rinsed out of the top section, following which this section, together with the attached electrode vessels, is filled with buffer to the levels *l*, *l'* (Fig. 2). The silver-silver chloride electrodes *E* and *E'* are then inserted, care being taken to avoid the trapping of air bubbles as the glass stopper *f* is seated, since this is to be the closed side of the system. To assure an ample supply of chloride ions for the electrode process, 25 ml. of approximately 1 *M* sodium chloride is allowed to flow slowly down each of the glass-enclosed silver tubes *t* and *t'* that also serve to make metallic contact with the electrodes. As the buffer around electrode *E*, for example, is thereby displaced, the liquid rises into the hollow stopper *p* and the excess overflows through *c*. While still filled with liquid the silver tube *t* is capped and the rubber tube *c* connected to the syringe *d* of the "compensator." This device, not shown, imparts a uniform movement at the desired rate to the piston of the syringe. Clockwise rotation through 90° of the stopcock *m* from the position shown in Fig. 2 then closes the right-hand side of the apparatus, connection with the compensator being retained.

On returning the center section to the center position (Fig. 3, c), sharp protein-buffer boundaries are formed at the junction of I and II on the right and II and III on the left. Injection of buffer from the syringe *d* then shifts the boundaries into view. In the 11-ml. cell 3.6 ml. per hour for 25 minutes will shift a boundary to the level H_i shown below in Fig. 12.

If the optical system does not provide for simultaneous registration of the pattern and its "base line" the latter can be recorded during emergence of the boundaries from behind the horizontal plates. The base line is the pattern obtained with no refractive index gradients in the channel and permits correction for imperfections in the channel and thermostat windows and the schlieren optics. Following interruption of the compensation and recording of the initial boundary positions (H_i of Fig. 12) current is passed from a battery or other suitable direct-current source. The current is measured potentiometrically as the electromotive force across a resistance of known value in series with the power source and the electrophoresis cell. If the current is not held constant its value is determined at intervals to permit computation of the quantity of electricity, in coulombs, that is passed. Since the initial boundaries are recorded with no current flowing it is preferable to interrupt the current for photography of the pattern that develops on electrophoresis.

III. Observation and Photography of the Boundaries

A. Schlieren Bands

The schlieren band optical system introduced by Tiselius (1) for the observation and photography of boundaries between colorless solutions is the simplest of those now in use and is shown diagrammatically in Fig. 4. Here a spherical lens L, the schlieren lens, forms at Y_0 an image of the illuminated, horizontal slit S, the source slit. The spherical camera lens O is focused on the cell, one channel of which is shown at T in the figure, and forms an image of the cell on the screen at G. If the fluid in the channel is homogeneous this image is uniformly illuminated. Suppose, however, that a protein-buffer boundary is present in the channel. Such a boundary consists of a region B of finite height in which the refractive index, n, varies continuously with the height, h, from a constant value in the homo-

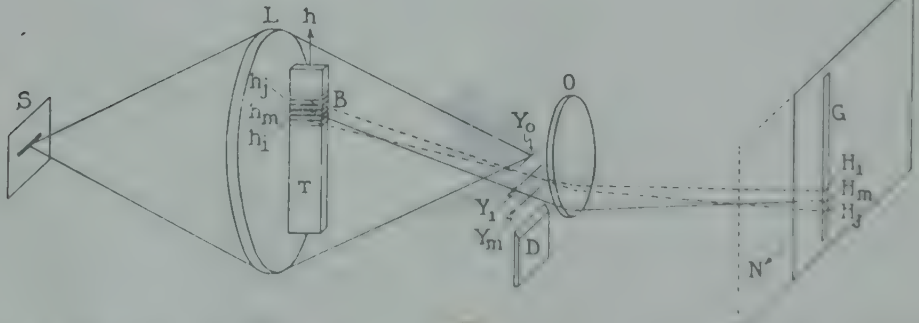

Fig. 4. Schlieren method for the study of refractive index gradients between solutions

geneous protein solution below the boundary to a constant, but usually smaller, value in the homogeneous buffer solution above. The gradient of refractive index, dn/dh, versus height is thus a bell-shaped curve, varying from zero to a maximum and back to zero. In Fig. 4 the variation of the gradient through the boundary is indicated by the density of the cross-hatching.

In terms of ray optics each layer of solution in the boundary acts as a prism and deflects the light passing through it. With but few exceptions, e.g. aqueous ammonia, the denser solution below the boundary has the higher refractive index and the deflection is downward. Considering the gradient in a sufficiently thin layer as constant, the light traversing this layer forms an image of the slit below the normal one at Y_0. In Fig. 4 two such displaced slit images are shown, that at Y_m being formed by light through the layer at h_m where dn/dh is a maximum whereas that at Y_1 is the superposition of two images formed by light through the layers at h_j and h_i, above and below h_m, respectively, where dn/dh has identical values, i.e. $(dn/dh)_m > (dn/dh)_i = (dn/dh)_j > 0$.

In terms of wave optics a wave front entering the channel as a plane normal to the optic axis would emerge with a sigmoidal profile, the plane front below the boundary lagging that above the boundary by $a\Delta n/\lambda$ waves. Here Δn is the refractive index difference between the two solutions forming the boundary and λ is the wavelength of the monochromatic light with which the slit S is illuminated. Actually the displaced slit "images" formed by all of the layers in the boundary combine to give a rectangular pattern of J horizontal interference fringes, the Gouy fringes (7), in the region from Y_0 to Y_m where $J = a\Delta n/\lambda$. These fringes, which are useful for diffusion studies (8, 9), are of little value for electrophoresis since they afford no indication of the position of the boundary in the channel.

If, now, an opaque screen D, called the schlieren diaphragm and having a sharp, horizontal upper edge, is placed in the image plane of L and raised to the level Y_m the light through the layer at h_m in the boundary is intercepted and a dark, narrow band or schlieren, i.e. shadow, appears at the conjugate level H_m in the image of the channel at G. On raising the diaphragm further, e.g. to Y_1, all light from h_i to h_j is intercepted and the dark band in the image of the channel broadens until the edges of the band are at H_i and H_j. This obviously can be continued until the undeflected rays at Y_0 are intercepted and the entire image becomes dark. If more than one boundary is present in the channel each will appear as a separate band over a range of diaphragm settings. However, if the minimum refractive index gradient between two overlapping boundaries exceeds the maximum gradient in a third boundary no diaphragm setting can be found at which all three appear as separate bands. Moreover, since gravity usually insures that the refractive index is uniform over a horizontal plane in the liquid, the corresponding coordinate in the image plane can be used to better advantage if it can be made to give the value of the gradient at each level in the channel. This is the purpose of the scanning and astigmatic modifications of the schlieren method described below.

B. The Scanning Camera

With a single boundary in the channel the series of fourteen photographs shown in Fig. 5, a was taken with the schlieren diaphragm raised a fixed distance between each exposure. The progressive broadening of the schlieren band is apparent. In the schlieren scanning procedure (10) the stepwise recording of Fig. 5, a is made continuous. The central portion of the image of the channel at *G* (Fig. 4) is masked by a vertical slit of 0.2-mm. width, say, and the photographic plate moved horizontally in the direction N at a uniform rate across this slit. Mechanically coupled with this plate movement is a steady vertical displacement of the diaphragm D. The resulting photographic positive of the boundary of Fig. 5, a is shown in 5, b and is called the boundary pattern. In conformity with common practice it has been turned, in Fig. 5, c, through 90° from the orientation in which it was recorded. The schlieren bands of Fig. 5, a thus correspond to equally spaced, narrow, horizontal sections of the complete pattern. If more than one boundary is present in the channel each appears as a separate peak in the complete pattern.

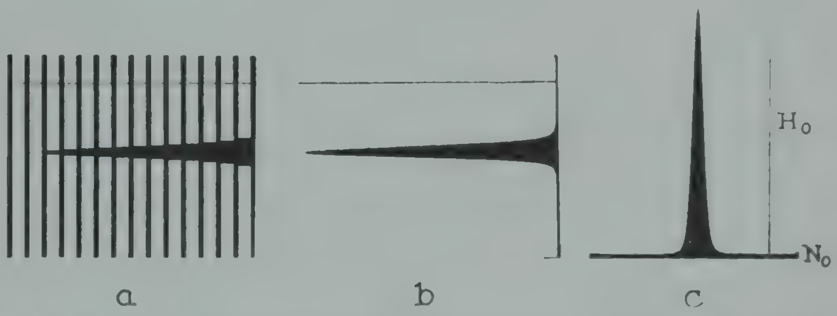

FIG. 5. Schlieren band and scanning photographs of a single boundary.

Since the deflection ΔY is proportional to the gradient producing it, and since the edges of a schlieren band are conjugate to the levels at which $dn/dh = \Delta Y/ab$, the constant ratio between the movement of the photographic plate and the schlieren diaphragm insures that the outline of the shadow in Fig. 5, c is a plot of $abM(dn/dh)$ as ordinate versus Eh as abscissa. Here a is the channel depth and b is the optical "lever arm," i.e. the distance from the center of the cell to the schlieren diaphragm after division of the length of each element in the path by its refractive index relative to air. M is the ratio of the plate movement to that of the diaphragm and E_h is the enlargement factor for the camera. The vertical line at H_0, Fig. 5, c, is due to a line ruled on the channel window and is taken as the origin of abscissa whereas N_0, the origin of ordinates, is the line, the so-called base line, joining those portions of the pattern conjugate to homogeneous columns of solution. In the ideal case the base line is straight and normal to H_0.

C. The Astigmatic Schlieren Camera

In the scanning process described above the complete pattern is not visible on the ground glass of the camera but only on the developed photographic plate. The astigmatic camera (11, 12) shown diagrammatically in Fig. 6 affords direct registration on the screen or plate of the curve of refractive index gradient versus height in the channel. Moreover, with the modification to be described in the following section this camera permits the registration of the refractive index directly as a function of the height with the aid of Rayleigh interference fringes.

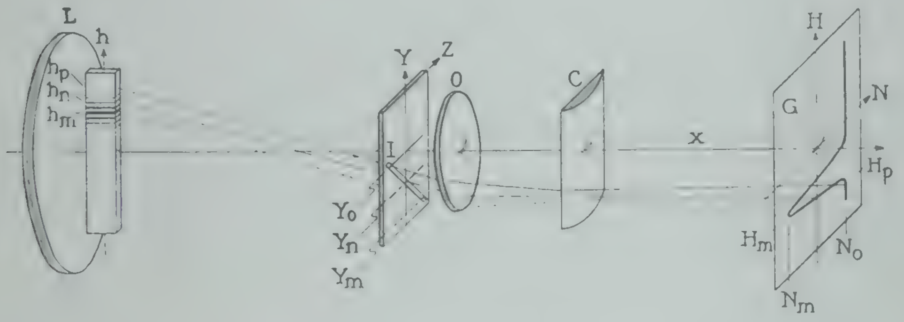

FIG. 6. The astigmatic camera.

In Fig. 6 the horizontal source slit, whose undeflected image is shown at Y_0, has been omitted from the diagram. The modifications of the optics of Fig. 4 consist in the replacement of the movable diaphragm with a fixed one having a slit I that makes an angle θ with the vertical and the addition of the cylinder lens C. This lens is mounted with its axis vertical and is focused, with the spherical lens O in position, on the plane of the schlieren diaphragm, i.e. its focus is such that a point source of light in the diaphragm plane becomes a sharply defined illuminated vertical line on the screen at G. Taken together, however, O and C constitute an astigmatic camera that reproduces the vertical coordinates of the channel and the horizontal coordinates of the diaphragm plane on the screen at G.

As before, if the fluid in the channel is homogeneous, all of the light passing through it converges to the normal slit image at Y_0. As can be seen in Fig. 6 only the light forming the extreme left-hand portion of this image passes the inclined slit. As the figure is drawn these rays lie to the left of the optic axis, x, and are deflected, therefore, to the right of that axis by the cylindrical lens, thus forming a narrow vertical band of light on the screen at N_0. This is the base line of the pattern. One undeviated pencil of light, that through the homogeneous layer of solution at h_p, is traced in Fig. 6 as a full line and forms one element of the base line at the conjugate level, H_p, in the image plane.

With a boundary in the channel, however, the light through the layer having the maximum gradient is deflected to Y_m. As is indicated by the dotted line in Fig. 6 only the rays forming the right-hand portion of this image pass the inclined slit. The spherical lens O brings these to focus at the same level H_m as in the absence of the cylinder lens but these rays pass the inclined slit to the right of the axis. Consequently they are shifted to the left by the cylinder lens and form the peak of the pattern at N_m.

The path of the light through another portion of the boundary is indicated by the dashed line and forms the corresponding element in the pattern. All other elements in the complete pattern are formed similarly. The focusing action of O on the channel insures that the vertical coordinate H of the pattern corresponds to h while that of the cylinder lens C on the inclined slit requires that the N coordinate depend upon the horizontal or cross-axis position Z at which the light passes this slit. As in the scanning procedure the proportionality between H and h is the enlargement factor E_h but must now be determined with C in position since the cylinder lens acts as a plane parallel glass plate with respect to the vertical coordinate and displaces the image plane slightly. The dependence of the N coordinate of the pattern upon the refractive index gradient in the cell is obtained by noting that the horizontal displacement $N - N_0$ depends, in part, on the angle θ that the inclined slit makes with the vertical. If Y_0 and Z_0 are the vertical and cross-axis coordinates, respectively, of the intersection of the normal slit image and the inclined slit

$$N - N_0 = E_z(Z - Z_0) = E_z(Y - Y_0) \tan \theta = E_z ab \tan \theta \, dn/dh$$

Here E_z is the enlargement factor, $\Delta N/\Delta Z$, between horizontal coordinates in the schlieren diaphragm plane YZ and the image plane HN.

D. Schlieren Diaphragms

As schlieren diaphragms one may also use a bar or phase plate instead of the straight edge or slit described in connection with Figs. 4 and 6, respectively. In the scanning procedure each of these is disposed horizontally, whereas with the astigmatic camera each is set at an angle to the horizontal. With an edge as schlieren diaphragm the pattern appears on the screen as a dark area in an illuminated field whereas with a bar or phase plate it is a dark line. With a slit the pattern appears as a light line on a dark field. The bars and slits in use, however, are of such width as to act essentially as two parallel straight edges, each of which produces Fresnel diffraction at the screen (13). The resulting uncertainty in the position of the line or edge on the screen is a limitation of the schlieren methods that has been partially overcome by the phase plate. This is due to Wolter (14) and is a glass plate with a transparent deposit on half of one face of such thickness as to retard the incident light by one half wave relative to the deposit-free half. For use as a schlieren diaphragm in electrophoresis and ultracentrifugation, Trautman and Burns (15) suggest placing a fine wire at the edge of the deposit. It is essential that the plate be of optical quality, with its faces flat and parallel.

E. Interference Optics

The schlieren methods considered above depend upon the deflection of the light by the gradients of refractive index in a boundary, whereas the interference methods that are coming into use in free electrophoresis depend upon the retardation of the wave front as it passes through the solution. Whereas the schlieren methods trace the variation of dn/dh with the height, it is the change of the refractive index n with h that is obtained from most of the interference fringe patterns.

In any interference method light from a common source is divided into two beams, one of which traverses the medium whose refractive index is of interest while the other traverses a homogeneous medium of invariant optical properties. The beams are then brought together and allowed to interfere, constructively if the path difference is an even number of half waves, destructively if this difference is an odd number. Two general methods are in use for division of the light into the two beams. In the Rayleigh interferometer the division is effected with two parallel slits in an otherwise opaque screen and is known as aperture splitting. In the Jamin, Mach-Zehnder, and Michelson interferometers the division is made with the aid of a half silvered mirror or partially reflecting air-glass interface and is termed amplitude splitting. In the Rayleigh interferometer the beams arrive at a common focus from different directions and interference is localized in this focal plane. In the amplitude splitting methods the beams are traveling in the same direction after reunion and the interference is not localized.

The simplest form that the Rayleigh interferometer takes for the study of free electrophoresis (16, 17, 18) is shown diagrammatically in Fig. 7. Here a point source of monochromatic light is focused on the plane at P

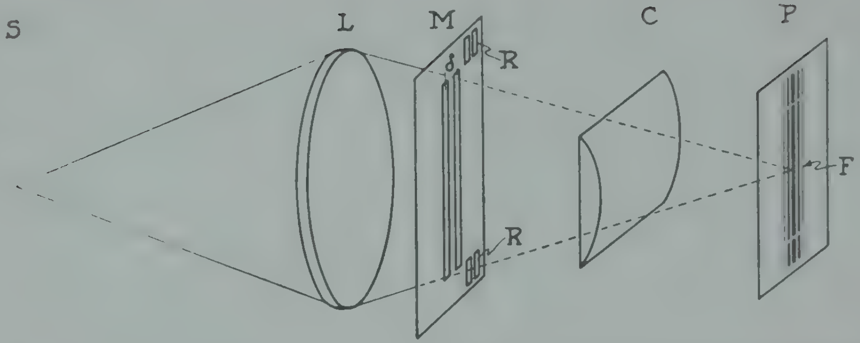

FIG. 7. Modified Rayleigh interference method for obtaining the refractive index as a function of the height in the channel.

by means of the spherical lens L. The cell mask M is placed near the cell, not shown, and is provided with two long vertical slits that allow light to pass through a channel in which a boundary may be present and a neighboring comparison "channel," respectively. In the Tiselius cell this comparison path is provided simply by extending the channel windows as shown at w-w' of Fig. 1, a. The cylinder lens C, whose axis is horizontal, focuses the cell vertically on P but does not affect the horizontal spacing of the Rayleigh fringes F that are formed at P as a result of the splitting of the incident light by the slits in M. L and C will be recognized as constituting an astigmatic camera that reproduces the horizontal coordinates of the source and the vertical coordinates of the cell in the plane at P.

If the fluid in the boundary channel is homogeneous the Rayleigh fringes are vertical with a distribution of intensity (19) along the horizontal or cross-axis represented by the continuous curve of Fig. 8. The ordinates of the dashed curve of this figure, which may be called the diffraction envelope, are proportional to the intensity in the source image with a single slit in the mask M. Whereas the fringes within the diffraction envelopes on either side of the central maximum are clearly visible with an ocular of moderate power, an exposure that is adequate for these faint fringes overexposes the central bright ones and they seldom appear in published fringe photographs. With narrow slits the width of the central diffraction envelope

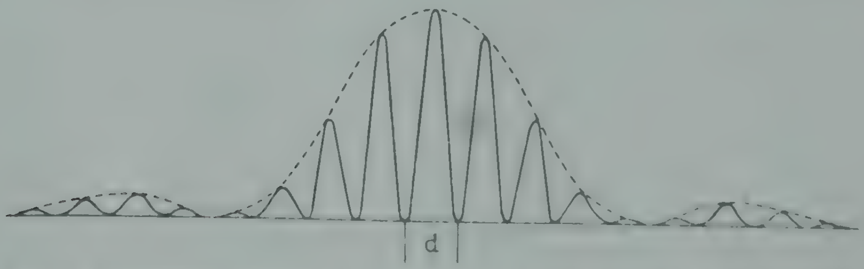

FIG. 8. Horizontal intensity distribution in the Rayleigh fringe pattern

varies inversely with the slit width, whereas the horizontal separation, d (Fig. 8), of the Rayleigh fringes is inversely proportional to the distance, δ (Fig. 7), between the centers of the masking slits. The relation is $d = b'\lambda/\delta$ where b' is the optical distance from M to P (Fig. 7). In the case of the 11-ml. cell, $\delta = 6$ mm. and, in a typical installation, $b' = 186$ cm. With mercury green light $\lambda = 5461$ Å and the fringe separation, d, is 169 μ. To obtain sharp fringes when the lens L is used at unit magnification, the horizontal dimension of the source should not exceed $d/4$. Although Fig. 7 is drawn with a point source, the vertical coordinates of this source are not imaged in the plane at P and this dimension is not critical. In fact a satisfactory source is obtained simply by rotating the schlieren source slit to the vertical position.

With one or more boundaries in the channel the fringes are warped into overlapping segments of the complete refractive index-height curve that intersect diagonally the central diffraction envelope. This is illustrated by the fringe patterns of Fig. 9. Since the path difference, in waves, between the solutions forming a boundary usually exceeds the number of vertical fringes within the diffraction envelope, these leave the envelope at the edges of the boundary and no one fringe traces the complete refractive index-height curve. A complete trace can be obtained, however, either by reducing the width of the masking slits or by using a grid of illuminated parallel slits as the light source (20). The decrease in intensity as the diffraction envelope is broadened by reducing the masking slit width renders this device impractical. The multiple slit source or raster overcomes this difficulty, but the resulting patterns contain the same information as that afforded by the use of a single source slit. The spacing of the raster slit images must match that, d, of the Rayleigh fringes.

As Svensson has emphasized (21) the cylinder lens functions at a relatively high aperture in the arrangement of Fig. 7. Since it is the most difficult element in the system to correct and has the shortest focal length of any of the lenses it is preferable to use it at a low aperture. If, in the arrangement of Fig. 6 the schlieren diaphragm is removed, the source slit is rotated to the vertical and the cell is masked by M of Fig. 7, the Rayleigh fringe pattern is obtained on the screen at G, only a small portion of the cylinder lens near its axis being used. This is the preferred arrangement.

With Jamin interferometry and monochromatic light (5, 22) the image of the channel is crossed by a series of horizontal fringes at positions conjugate to successive levels at which the optical path differs by one wavelength. Since both the horizontal and vertical coordinates of the channel are focused, this is the system of choice for the study of nonplanar boundaries. It does not lend itself readily, however, to the evaluation of the refractive index at levels between widely spaced fringes conjugate to weak

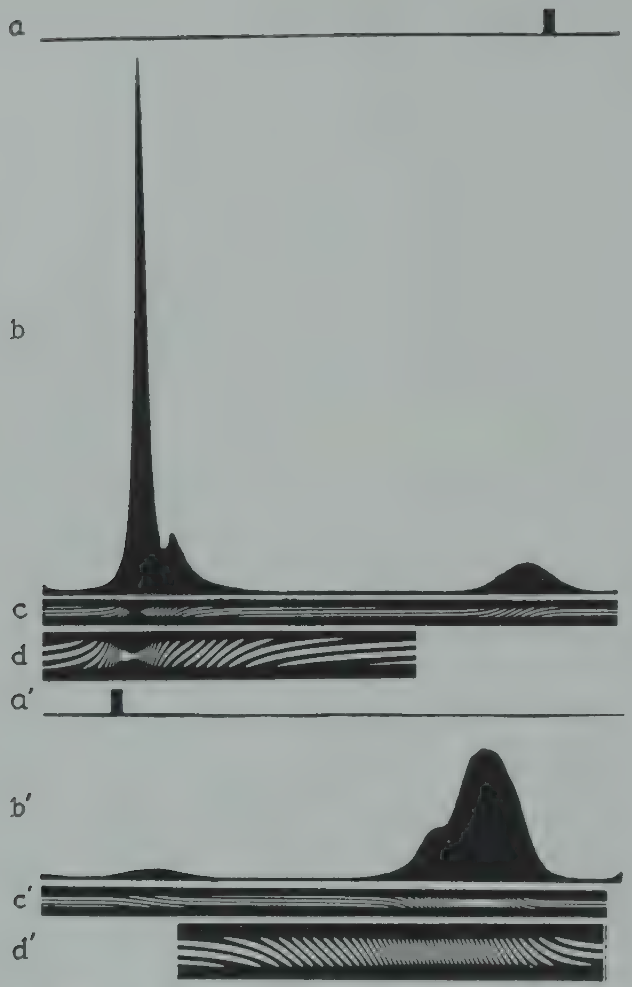

FIG. 9. Schlieren scanning, bb′, and Rayleigh fringe patterns, cc′, obtained with autocollimating camera and 2-ml. cell on free electrophoresis of 0.76% ovalbumin in 0.1 Γ/2 sodium phosphate buffer at pH 6.8 for 7500 seconds at 8.8 volt/cm. Initial boundaries shown in aa′. Rayleigh fringes conjugate to moving boundaries shown enlarged in dd′. Primed letters refer to pattern in which boundaries descend.

gradients. With white light (4) the second Jamin plate is tilted, with the aid of a graduated drum and screw, to equalize the path difference at a given level and thus bring the only usable fringe, the white, zero-order fringe, into coincidence with the cross hairs of an eyepiece focused on this level.

F. The Parallel Light Modification

In the case of the schlieren lens, *L* of Fig. 4, the requirements are those of a telescope objective. Since this lens must have a diameter of 4 inches to cover the standard Tiselius cell, one having more than two elements, i.e. an achromat, is hardly practicable and with such a lens the image quality deteriorates at apertures greater than $F/9$. Consequently this lens should have a focal length of 36 inches or greater, a consideration that largely determines the over-all dimensions of the electrophoresis equipment. Thus if both L and O of Fig. 6 are used at unit magnification the length of the supporting optical bench is about 18 feet. Two modifications of the basic optical system are in current use, both of which permit a reduction in length without seriously affecting the image quality. In the parallel light modification (23) both windows of the thermostat in which the electrophoresis cell is immersed are provided with identical achromats, termed the collimating and field lenses, respectively, with the distances from source to collimator and from field lens to schlieren diaphragm both equal to the focal length. With a point source the light falling on the cell is then parallel instead of convergent as in Fig. 6.

G. The Autocollimating Modification

In the autocollimating modification (5, 23, 24), shown diagrammatically in profile in Fig. 10, a single schlieren lens L is made to serve both as collimator and field lens by placing the channels, T, of the cell almost in contact with a first surface mirror M so that the incident light is reflected back through the cell and lens. The double passage of the light through the cell doubles the optical sensitivity since the effective channel depth is now $2a$. Although the distortion of the gradient curve arising from the finite depth increases as the square of this dimension, this is not as serious with double passage through a depth a as with single passage through $2a$ since the mirror may be tilted to effect partial compensation. Thus the maximum in the recorded gradient curve of a sharp boundary is depressed somewhat since the rays forming this portion of the pattern curve out of the layer of solution in the boundary where the gradient is a

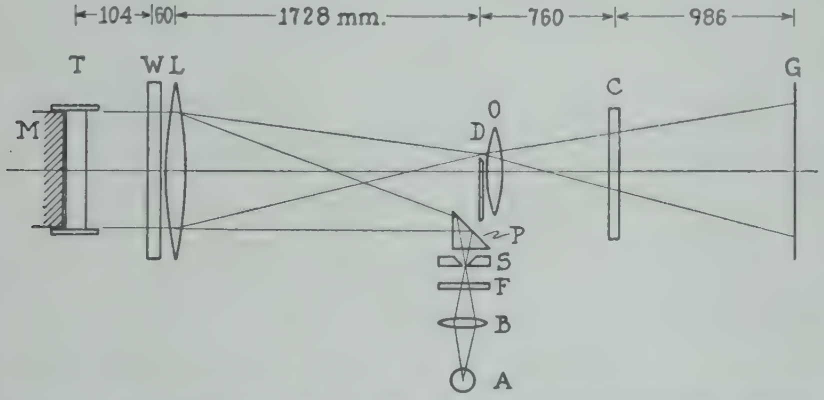

FIG. 10. Autocollimating schlieren and Rayleigh interference system.

maximum (25). This depression is minimized if the entrance and exit angles of these rays on first passage are equal in magnitude and opposite in sign and by tilting the mirror the rays may be returned over this same path.

For schlieren photography the light is brought in from below, as indicated in Fig. 10, with the upper edge of the prism P just clearing the lower edge of the camera lens O. Since it is essential for the incident and reflected light to be as nearly "on axis" as possible it is not advisable to convert from the schlieren to interference optics simply by rotating the source slit through 90° as is done in the case of a single passage system. For interference work the light is brought in from the side, i.e. the A-S axis of Fig. 10 is normal to the plane of the paper. This is accomplished either with a second source slit assembly or by rotation of the entire assembly from the position shown to the horizontal axis.

In the autocollimating system the mirror is the object plane of the camera lens O. The first passage of light through the cell results in the formation, by the mirror, of an image of the cell that is as far to the left of M as the real cell is to the right. In order to have the cell and its image in equally good focus the reflecting surface of the mirror is thus the best compromise as object plane. Owing to the presence of the schlieren lens between this plane and the camera it is the virtual image of this plane formed by the lens L upon which the camera is focused. Although it would be preferable to have the reflection at the inside surface of the left-hand channel window, this has not proved practicable.

H. Optical Specifications

Although Fig. 10 is not drawn to scale, the spacing of the elements in a representative installation is given above the figure. The following specifications for the elements are also typical. The schlieren lens L is a coated 4-inch achromat of 68 inches focal length and is corrected spherically for focus at infinity. The spherical camera lens O is a 2-inch achromat of 36 inches focal length, while the cylinder lens C is a 3-inch achromat of 17 inches focal length. Both O and C are corrected for use at unit magnification. The chromatic correction of all lenses is for the mercury green and yellow, that of the camera objective O making allowance for the water in its object space. The faces of the thermostat window W are flat to $\frac{1}{4}$ wave and parallel within 20 seconds of arc. The dimensions of the mirror M are 40 × 85 × 12 mm. and the reflecting surface is flat to $\frac{1}{4}$ wave. Unprotected rhodium has proved to be satisfactory as the reflecting deposit for use under water. A spare mirror is kept on hand as replacement in the event the one in use becomes scratched or has to be returned for recoating.

I. Adjustment of Optical System

Although servicing facilities are available for most commercial electrophoresis equipment, the following suggestions for the adjustment of the optical system may help the investigator in recognizing difficulties that arise. With the thermostat filled and regulating at 1°, say, and with the mirror adjusted to reflect light into the camera, the rulings on a precision glass scale are placed almost in contact with the reflecting surface. With the cylinder lens removed, the spherical lens O and plate carriage G are adjusted to give sharply defined images of the rulings in the plane of the photographic emulsion at approximately unit magnification. Comparison of the spacing of these images with the rulings gives the vertical enlargement, E_h, for the schlieren band and scanning camera.

It may be noted that practice differs as to the plane on which the camera is focused. Svensson has suggested (25) that the Wiener skewing can be minimized by focusing at a distance $a/6$ to the right of the center of the channel in the single-passage system (Fig. 4). With double passage (Fig. 10), the equivalent plane is a distance $a/3$ to the right of the left-hand channel window. In any case the scale must be at a position corresponding to the center of the cell, i.e. the mirror in the double passage system, for the evaluation of E_h.

A useful accessory in focusing and in work with Rayleigh fringes is a Ramsden ocular, Gaertner L372, focused on cross lines, Bausch and Lomb 31-16-30, and mounted, with a graduated vertical movement, in a carriage that is interchangeable with that for the photographic plate. Since the cross lines reproduce accurately the plane of the emulsion, a scale line image that is focused in the ocular will also be focused on the emulsion, thus avoiding the loss of image detail characteristic of a ground-glass screen. If the thickness of the glass on which the scale is ruled is twice that of a channel window their effect in shifting the focal plane is duplicated by the scale.

The cell, filled with water, is next substituted for the scale and centered on the optic axis. Vertical and horizontal adjustments of the camera lens O and plate carriage G, but with no displacement along the optic axis, now position properly the cell image at G. With the camera thus aligned the source slit assembly, consisting of the lamp A, the condensing lens B, the filter F, the adjustable slit S and the prism P, is moved parallel to the optic axis until the source slit is focused on the schlieren diaphragm. This adjustment is made by observing the channel image on a ground glass at G as the horizontal edge of the diaphragm is advanced through the image of the source slit, a test that is also useful in detecting other imperfections (26). Suppose the width of the slit S is 0.05 mm. and the edge of the diaphragm is just below its image. At unit magnification a perfect image of the slit would also have a width of 0.05 mm. and movement of the diaphragm through this distance would cause the image of the channel to become dark uniformly. With correct slit focus and good optics the image goes dark with a diaphragm movement of 0.06–0.08 mm. If a greater movement is required and the image darkens progressively from top to bottom, or conversely, the slit is incorrectly focused. If a large movement is required and the image darkens uniformly the slit image is not parallel to the edge of the diaphragm. Following the focusing of the source slit the mirror is oriented so that undeflected light enters the camera lens, O, 15–20 mm. above the optic axis. Steeper gradients can then be recorded than if only the lower half of O were used.

Another test for camera focus, one that is peculiar to the schlieren method (23) and extremely sensitive, may be illustrated with the aid of Fig. 11. Here rays, equally spaced in the object plane C at the center of

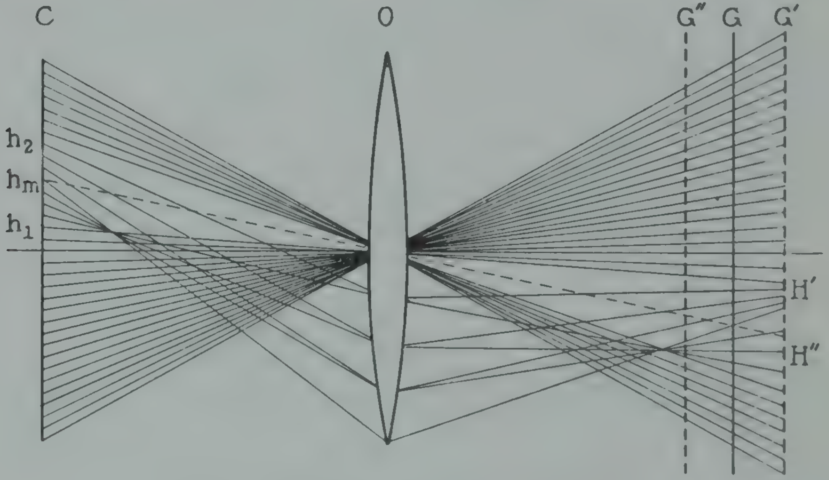

FIG. 11. The halo test for camera focus.

the cell to indicate uniform illumination, have been traced through an idealized system. If a boundary is present in the region from h_1 to h_2, with the maximum gradient at h_m, the rays through this region are deflected as shown but, in the absence of a diaphragm the illumination remains uniform in the image plane at G. In a neighboring plane at G', however, the illumination is above the average at H' and below at H'', whereas this order of intensities is reversed in the plane at G''. With a sharp boundary in the channel and the schlieren diaphragm removed, the cell image is uniformly illuminated only in the focal plane of the camera. The test is most sensitive with a sharp boundary in the cell, i.e. one for which Δn is small and the maximum gradient, n_m', large. Even with n_m'

so large that the camera lens mount acts as a schlieren diaphragm a "halo" appears above, or below, the schlieren band on the screen at *G* if this is moved by as little as 1 mm. to the right or left, respectively, of the focal plane.

The final step is the positioning of the cylinder lens *C* (Fig. 10). To facilitate this the schlieren diaphragm is replaced by a metal sheet with a series of pinholes on a horizontal line at the optic axis. With this sheet diffusely illuminated with white light the cylinder lens, centered on the optic axis and normal to it, is moved along this axis until a sharply defined grid of illuminated lines is formed on the ground glass or, preferably, in the Ramsden ocular. The axis of the cylinder lens is vertical when this grid is parallel to a plumb line and a comparison of the horizontal spacing of these lines with the spacing of the pinholes gives the horizontal enlargement, E_z, of this astigmatic camera. The vertical enlargement, E_h', of this camera is most readily obtained with the glass scale at the mirror and the double slit mask, *M* of Fig. 7, so placed that the rulings are in the light path to one slit only. The vertical Rayleigh fringes in the image plane of the camera are then interrupted at levels conjugate to the rulings.

J. Apparatus Constants

In using the schlieren methods, especially the parallel light and autocollimating modifications, the determination of the optical lever arm, *b*, has proved to be difficult. If the light through the thermostat is accurately parallel, *b* is simply the focal length of the field lens and can frequently be obtained from the manufacturer. Since the principal planes of most achromats are within the glass, the distance from the crown of the lens to the diaphragm is thus less than *b*. This constant does not appear in the interference procedures, however, and these can be used in its evaluation as follows (24).

A Bausch and Lomb ophthalmic prism, PR-5-005, is cut in two, normal to its base. One half is placed, base down, almost in contact with the mirror in the path of the light to one slit in the cell mask while the other half is placed at the same level, but with base up, in the path of the light to the other slit and the resulting fringes photographed. Since a prism corresponds to a gradient of constant value the fringes conjugate to the prisms are uniformly spaced diagonal ones (27). The split-prism arrangement described here equalizes the path at the center of the diagonal fringe system and thus avoids the fuzziness that results from the excessive path difference if only one prism is used. Using the vertical fringes on either side of those conjugate to the prisms to align the plate in the comparator, the number of diagonal fringes per unit height, $\Delta j/\Delta H$, is determined and divided by E_h' to obtain $\Delta j/\Delta h$. If, now, the source slit is turned so that its image is horizontal and the prisms are placed serially, with both bases down, almost in contact with the mirror, the displacement, ΔY, of the slit image may be measured and $b = (\Delta Y/\lambda)(\Delta j/\Delta h)$.

The channel depth a is determined with the aid of a small brass bar whose length l is slightly less than a and whose ends are flat and parallel. With the channel on the stage of a microscope so that the a-axis is vertical, the bar is inserted and the difference $a - l$ determined with the aid of the graduated fine adjustment of the microscope by focusing alternately on the upper end of the bar and the inside wall of the channel window. This is repeated as the bar is shifted along the channel.

The average cross-sectional area, s, of a channel is required for computations of the mobility and is obtained from the volume of each side of the center section, as computed from the weight of mercury recoverable from a channel filled therewith and the height of this section as determined with a vernier caliper. This is an average, however, over the entire height, including the horizontal plates. The channels of most center sections have a uniform cross section except where they pass through the end plates (28). Correction for this is made by subtracting the volume of a channel in these plates from the total volume and dividing the difference by the total height less the thickness of the plates. The thickness, width, and depth of the slot in an end plate can be measured with the aid of a micrometer, feeler gauge, and inside vernier caliper, respectively.

IV. Analysis of Electrophoretic Patterns

A. The Apparent Composition

If the pattern has been recorded with one of the schlieren methods the analysis may be made with the aid of an enlarged tracing, such as that of Fig. 12 for a human plasma. If the separation of the boundaries is incomplete, as is the case in this figure, one must make a more or less arbitrary separation in order to assign a definite area to each peak. This separation may be made by drawing ordinates from the minima between adjacent peaks (29), the dashed lines of Fig. 12, or by constructing bell-shaped curves (30), indicated by the light lines in the figure, such that the sum of their ordinates is equal to the observed value at each level. Although several of the plasma proteins that are distinguishable on free electrophoresis, i.e. albumin, α_1-, α_2-, β-, γ-globulin, and fibrinogen, ϕ, are known to be inhomogeneous with respect to such properties as size, shape, and even mobility, the resolution of Fig. 12 into a larger number of peaks is not justified since asymmetry can arise from the pH and conductivity gradients in a boundary (31) as well as from an asymmetrical distribution of mobilities. While it is known that the apparent composition, as determined by the use of ordinates through the minima, changes somewhat as the boundary separation increases, no measurements have been made to establish which method of area assignment is least dependent on this separation. The greater precision that is possible with interference optics suggests their use in such comparisons.

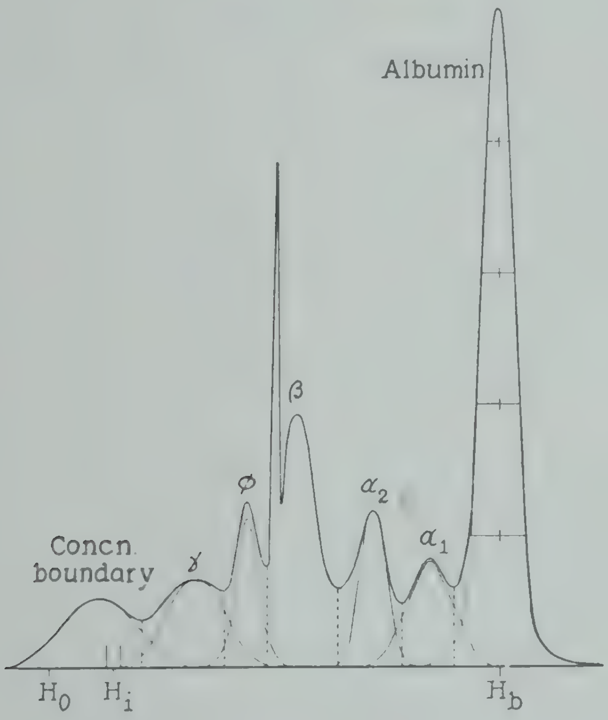

FIG. 12. Tracing of the electrophoretic pattern of a human plasma.

The apparent composition is obtained as the ratio of the area of a peak, measured with the aid of a planimeter, to that of the entire pattern exclusive of the concentration boundary. This procedure assumes that at a boundary there are no concentration gradients other than those of the protein disappearing therein and that all of the proteins have the same specific refractive increment.

The uncertainty in the analysis due to the assumption of constant refractive increments depends largely on the nature of the proteins in the mixture. If these are lipid- and carbohydrate-poor proteins the increment is approximately independent of the protein. This is indicated by the data of Table I for some representative proteins (32, 33). The specific refractive increment, k, of a protein is defined as $k = (n_{\text{solution}} - n_{\text{solvent}})/p$ where the concentration p is expressed in grams dry weight of protein per 100 ml. of solution. On this scale k is independent of protein concentration over a range of several per cent or more and varies only slightly with the concentration of a neutral salt. At pH values other than the isoelectric pH the increment includes the contribution of the counterions and increases somewhat with the net charge on the protein. In Table I the lipid content

TABLE I

Specific Refractive Increments of Isoelectric Proteins at 0°C. and $\lambda = 5780$Å

Protein	k_0	%N_2
Bovine serum albumin	0.00190_1	16.05
Human serum albumin	0.00188_7	15.95
Ovalbumin	0.00187_6	15.72
β-Lactoglobulin	0.00186_5	15.53
Human γ-globulin	0.00187_5	15.9
Human α_1-lipoprotein	0.00181	8.0
Human β_1-lipoprotein	0.00174	4.2

of the lipoproteins is indicated by the low percentages of nitrogen in these materials (column 3), but the depression of the increment, k, is not excessive. The increments are dependent on both the temperature and the wavelength and those of Table I for the lipoproteins have been increased by 3×10^{-5} over their values at 20°C.

The uncertainty in the analysis resulting from the superimposed gradients at a boundary has been considered in terms of the available theory in the preceding chapter. The results of some experimental studies are summarized in Fig. 13. Here the apparent relative concentrations of serum albumin in three mixtures containing this protein, and of the A_1 component of ovalbumin, are plotted as ordinates against the ratio of total protein concentration to the ionic strength, i.e. $2p/\Gamma$ as abscissa, a scale suggested by equation (29) of the preceding chapter. The unfilled circles represent values from the patterns of rising boundaries, the filled circles from descending boundaries, and the crosses are mean values from a pattern pair. In the case of human plasma (34) and the albumin-globulin mixture (35) the range of $2p/\Gamma$ was insufficient to establish a significant difference between the values from the two patterns. The ovalbumin results (36) were obtained at constant ionic strength, those for the hog serum (37) at constant total protein concentration, whereas both p and Γ were varied in the other systems. Although more scattered, owing presumably to the inferior resolution of the descending boundaries, the filled circles for the hog serum indicate a lower slope than the unfilled ones, as required by the theory. In the case of the albumin-globulin mixture the extrapolated value agrees with the known composition within the limits of error of the measurements and the theoretical uncertainty in the extrapolation. The relatively steep slope in the case of ovalbumin illustrates the considerable departure of the apparent from the true value when the protein mobilities are not very different.

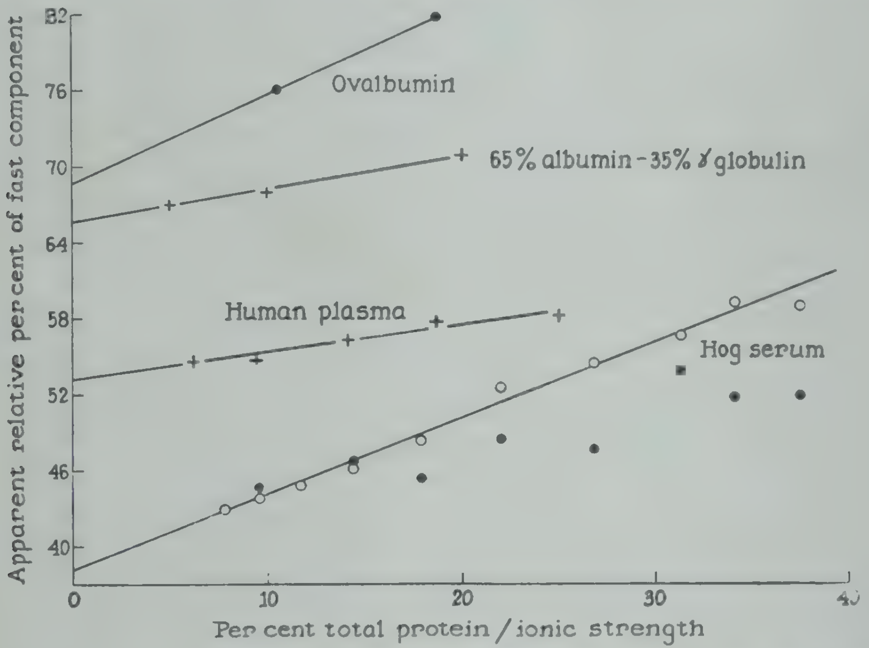

FIG. 13. Dependence of the apparent composition of protein mixtures on the ratio of protein concentration to ionic strength.

B. *Mobility Measurements*

It is also convenient to use the pattern tracing (Fig. 12) in the evaluation of mobilities. If, as in the case of the albumin boundary of the figure, the gradients are symmetrical about the ordinate through the maximum the mid-points of a series of chords will lie on this ordinate and it may be taken as the boundary position, H_b. The distance through which the boundary has moved is then $\Delta h = [(H_b - H_0) - (H_i - H_0)]/E_h E_t$. Here E_t is the tracing enlargement whereas the initial boundary position, H_i, is taken as the center of the schlieren band that is recorded, with a large diaphragm displacement, after interruption of the compensation but before passage of the current. As is clear from the figure, it is incorrect to assume that the concentration boundary does not move during electrophoresis and to use its position as that of the initial boundary.

If the mid-points of the chords do not lie on the ordinate through the maximum the boundary is skew and its correct position is that of the first moment or centroidal ordinate of the gradient curve (13). In terms of the cell coordinates and for an isolated boundary this position is defined as

$$\bar{h} = \frac{\int_0^\infty h(dn/dh)dh}{\int_0^\infty (dn/dh)dh}$$

where the denominator will be recognized as the area of the gradient curve. Several methods for the evaluation of $\bar{h}$ are available (38), but the highest accuracy is attained in their use by direct observation of the photographic plate with the aid of a two-coordinate comparator. With either an edge, slit, or bar as schlieren diaphragm the Fresnel diffraction at the plate can be used to advantage (13, 39) in the evaluation of dn/dh as a function of the height.

In the case of a skew boundary the ordinate bisecting the area of the gradient curve lies between the centroidal ordinate and that through the maximum gradient. The bisecting ordinate may be located by trial and error on the tracing with the aid of the planimeter and affords a more nearly correct location of the boundary than does the maximum ordinate.

If κ_p is the specific conductance of the protein solution and s the cross-sectional area of the channel, a current of I amperes corresponds to a field strength $E_p = I/\kappa_p s$. If the current remains constant for the Δt seconds in which the boundary moves Δh centimeters, its velocity is $\Delta h/\Delta t$ and the mobility of the protein ions disappearing therein is

$$u_p = \Delta h/E_p\Delta t = \kappa_p s\Delta h/I\Delta t \tag{1}$$

Here the denominator is the number of coulombs passed in causing the boundary to sweep through the volume $s\Delta h$ and is obtained from a current-time curve if the current varies. If v is the volume swept through per coulomb $u_p = v\kappa_p$.

Since the conductance, κ_p, is measured at 0°C., as described in a subsequent paragraph, the mobility that is computed with the aid of equation (1) is the value at this temperature although the boundary displacement occurs at a somewhat higher temperature. No significant error is made in this procedure since the displacement depends on mobility ratios and these are insensitive to temperature.

Unless information is available as to the conductances of the new solutions formed during electrophoresis, the only true mobility obtainable from a pattern is that of the protein disappearing across the leading boundary descending into the end protein solution. The mobilities computed from the displacements of the other boundaries with the aid of equation (1), thereby assuming the same field strength in the new solution from which the protein disappears as in the end protein solution, are apparent values

C. Fringe Analysis

Although the Rayleigh fringe patterns do not lend themselves as readily to graphical analysis as do the schlieren patterns the information contained therein is more accurate. This is due largely to the absence of diffraction effects and the fact that a minimum of optical density may be located more reproducibly than the edge of a shadow. Although interferometry in free electrophoresis has been available for several years now, it has not found the application that its precision warrants. This may be due, in part, to the need for a comparator, preferably one having graduated movements at right angles and a stage that can be rotated, for the fringe analysis.

The procedure depends somewhat upon the character of the fringes that are obtained with water in the channel. If these "base line" fringes are straight within 0.1 of their separation, d of Fig. 8, and the allowable error in n is 2×10^{-6}, i.e. one-tenth of a wave in the standard cell, no correction is necessary. Most cells now available meet this requirement. The pattern of the boundary system is aligned in the comparator with the fringe envelope parallel to a comparator axis and a central minimum at one end of the pattern focused on the fixed cross hairs of the screen or ocular. If a complete description of the pattern is desired the comparator readings, H_j, at which each successive diagonal minimum, j, is centered on the cross hairs are tabulated. Since each diagonal fringe represents a constant refractive index increment of $\Delta n = \lambda/a$ such a tabulation lends itself readily to the various procedures for numerical differentiation and moment analysis that are available (38). A plot of the readings against the fringe number j gives the refractive index as a function of the height whereas a plot of the reciprocal of the fringe separation, $1/(H_{j+1} - H_j)$, against the mean position, $(H_{j+1} + H_j)/2$, gives a curve approximating closely to the gradient curve. This is illustrated in Fig. 14 where the circles represent the reciprocals of the fringe separations and the full curve is a trace of the schlieren pattern of the same boundary system.

For an analysis it is unnecessary to prepare a plot or even to tabulate all H_j values. A minimum in the gradient curve corresponds to a maximum in the vertical separation of the diagonal fringes. The number, j_m, of the fringe at which a maximum occurs can be determined by numerical interpolation (40). Thus, if $\Delta H_1 = H_j - H_{j-1}$, $\Delta H_2 = H_{j+1} - H_j$ and $\Delta H_3 = H_{j+2} - H_{j+1}$, where ΔH_2 is a maximum, i.e. $\Delta H_1 < \Delta H_2 > \Delta H_3$, the value of j at the point of inflection in the curve of j versus H is

$$j_m = \frac{(2j+1)\Delta H_2 - j\Delta H_3 - (j+1)\Delta H_1}{2\Delta H_2 - \Delta H_1 - \Delta H_3}$$

In the pattern from which Fig. 14 was derived the separation of fringes 43 to 46 was 466, 532, and 493 μ, respectively, and $j_m = 44.6$. Other separations are maximum at $j = 50.4$, 56.7, 69.0, 92.0, and 102.0, the last interval representing the protein concentration boundary. Thus the apparent composition of this serum is 44.6/92.0 or 48.5% albumin, 6.3% α_1-, 6.8% α_2-, 13.4% β-, and 25.0% γ-globulin.

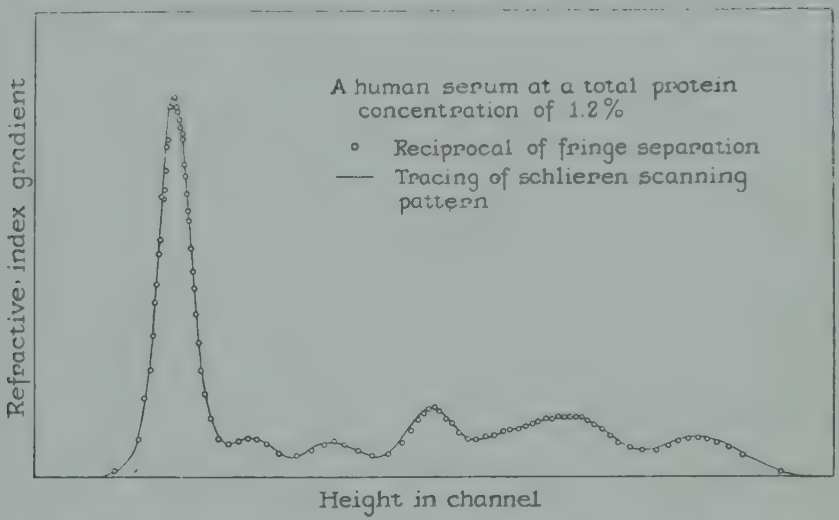

FIG. 14. Comparison of a schlieren scanning pattern, ——, with the reciprocal separation of the Rayleigh fringes, O.

If the solutions above and below a boundary are homogeneous the fringes conjugate thereto are vertical. Since the refractive index difference between the two solutions does not correspond, in general, to an integral value for $j = a\Delta n/\lambda$, these vertical fringes are shifted horizontally with respect to each other and the ratio of this shift to the separation, d of Fig. 8, is a measure of the fractional part of j. This shift is measured with the aid of the cross-axis movement of the comparator, a movement that may also be used to correct for any departure of the base line fringes from linearity (41).

If the windows of a channel are not parallel, a prismatic effect appears. Since water is the liquid in the comparison path the fringes are vertical with this fluid in the boundary channel, whereas with a homogeneous solution in this channel they remain straight but are now inclined slightly with respect to the fringe envelope (27). Alignment is facilitated if reference fringes are formed above and below those conjugate to the channel. The light through auxiliary slits, R, in the cell mask, M of Fig. 7, bypass the cell and thus form the desired reference fringes.

V. Preparation of Solutions for Electrophoresis

A. Buffer Solvents for Electrophoretic Analyses

The "best" buffer solvent for the analysis, by means of free electrophoresis, of a given mixture of proteins is generally considered to be one in which the proteins are stable, in which a maximum number of clearly separated boundaries is obtained, and in which deviations of the pattern pair from enantiography are minimal. As noted in the preceding chapter, high buffer capacity and low buffer ion mobilities favor enantiography, as does a pH at which all protein components have mobilities of the same sign. In the search for a best buffer much of the work has also been predicated on the assumption that the binding of small ions other than hydrogen, and of small molecules, by the proteins should be avoided. For example, 0.1 M sodium diethylbarbiturate (NaV) at pH 8.6 has been widely used for the electrophoretic analysis of human sera and plasmas (6). Of the more common buffer solvents studied, this one gave the best resolution of the boundaries due to the protein components and the pattern pair was most nearly enantiographic. The recent work of Aronsson and Gronwall (42) on the paper electrophoresis of serum in a boric acid-trishydroxymethyl amino methane buffer containing ethylenediaminetetraacetic acid suggests, however, that a larger number of serum proteins can be thus identified than in conventional buffers. The use, in the buffer solvent, of complexing agents specific for fractions of the proteins in one or more of the groups designated as albumin, α_1-, α_2-, β- and γ-globulins may very well increase the utility of free electrophoresis as an analytical tool and will doubtless be investigated further. In using polycomponent buffers, however, the patterns can be most readily interpreted if enantiography is preserved and evidence is educed that new boundaries that may appear are not moving concentration boundaries due to the presence of more than two species of small ions in both end solutions.

B. Dialysis

In the case of serum it is common practice to dilute 5 ml., say, of this material with 10–15 ml. of buffer and dialyze at 0–6°C. against two 250-ml. portions of the buffer for 1 day each followed by an additional day of dialysis against a third portion of sufficient volume to fill the cell and electrode vessels, about 1500 ml. in the case of the apparatus of Fig. 2. This procedure eliminates from the serum virtually all diffusible solutes other than the buffer electrolytes. In the case of *normal* sera Svensson and Olhagen (43) have tested an alternative procedure based upon the approximate constancy of both the nature and concentration of the diffusible solutes from one individual to another. They suggest two buffers, A and B, such that when one volume of serum is mixed with three volumes of A, a solution is obtained whose electrolyte composition is essentially that of buffer B and these are used without dialysis. For A they recommend 0.1333 C NaV, 0.0133 C HV, whereas B is 0.1 C NaV, 0.01 C HV, 0.019 C NaCl, 0.007 C $NaHCO_3$, 0.00125 C KCl. Except for a larger refractive index difference across the protein concentration boundary δ, the patterns thus obtained are in agreement with controls in which dialysis preceded electrophoresis. The enhanced δ area could arise from the nonelectrolytes in serum, e.g. glucose, or from the contribution to the regulating function of the ions neutralizing the charge on the protein or from both. It should be possible, however, to modify the buffer B further to eliminate the concentration boundaries.

C. Clarification of Protein Solutions

In many naturally occurring mixtures of proteins, e.g. lipemic sera and milk whey, particles or globules are present in suspension having sizes comparable with the wavelength of visible light and thus scatter this light. The resulting opalescence of the solution interferes with schlieren or interference photography and clarification is required. High speed centrifugation is partially effective but complete clarification can sometimes be obtained only at speeds that also cause sedimentation of the proteins. Preliminary dialysis against an appropriate electrolyte solution may alter the properties of the particles so as to facilitate their sedimentation (44). Filtration through fine filters is also partially effective but is accompanied by the tendency of the filter pores to become clogged. A sintered glass filter specially designed for the clarification of protein solutions was employed in light scattering measurements (45). In the procedure suggested by Lindquist (45a), which may be termed clarification dialysis, this tendency has been minimized. The circular membranes of a multicompartment dialyzer that rotates on a horizontal axis are replaced with ones having pores that will pass the proteins but not the particles and alternate compartments half filled with opalescent protein solution and buffer, respectively. Except for some residual osmotic flow the clarification results from diffusion of the proteins through the membranes. If clarification is not practicable, the effects of the scattering can be minimized by using red, or infrared, light from an incandescent source and a fast, red-sensitive photographic emulsion of wide latitude (46, 47).

D. Buffer Solvents for Mobility Measurements

In mobility measurements on pure proteins over an extended range of pH more than one buffer system is used and their selection involves among other things, a consideration of buffer capacity. As defined by Van Slyke (48) the capacity, β, of a weak acid HR is

$$\beta = 2.3 \frac{(\mathrm{R}^-)(\mathrm{HR})}{(\mathrm{R}^-) + (\mathrm{HR})}$$

For a given concentration of the R constituent, i.e. $(\mathrm{R}^-) + (\mathrm{HR})$ = constant, this capacity is a maximum at pH = pK, where $(\mathrm{R}^-) = (\mathrm{HR})$, and decreases to one-third of the maximum value at pH = p$K \pm 1$. Most curves of mobility versus pH are obtained, however, at constant ionic strength, i.e. (R^-) = constant, and in this case the capacity increases monotonely with decreasing pH but not in proportion to the concentration of undissociated acid. Thus at pH values much greater than p$K + 1$, and constant ionic strength, the buffer capacity is frequently inadequate whereas at pH $\leq$ p$K - 1$ the concentration of weak acid is relatively high. High concentrations of such solutes affect adversely the temperature of maximum density of water and alter the aqueous character of the medium. For example, a 0.1 M acetate buffer at pH 3.6, where the concentration of undissociated acid is 1 M, could hardly be considered an aqueous medium. Thus a buffer system is seldom used to cover a pH range of more than one unit on either side of its pK.

Specific buffer effects on the protein mobility are reduced by substituting, at a given ionic strength, a neutral salt, usually sodium chloride, for a portion of the buffer salt. Moreover, in mobility measurements a low concentration of protein is used, and if the ionic strength is not too low, as much as 90% of this can be contributed by the neutral salt. In fact, it is possible to dispense with the buffer electrolytes completely (49) and use the buffer capacity of the protein to control the pH. In this case the pH of the protein in a neutral salt solution is adjusted to the desired value with small amounts of strong acid or alkali and dialyzed against a large volume of the salt solution whose pH has been similarly adjusted. Owing to the relatively low buffer capacity of the protein it must be present at an appreciable concentration if the pH in the body of the protein solution is to remain constant during electrophoresis.

In the case of proteins that are available as salt-free solids it is also possible to prepare a solution of known electrolyte composition without recourse to dialysis. Although dialysis is almost universally used in the preparation of proteins for electrophoresis, in so doing the investigator has lost a measure of control over the system. As was noted in the preceding chapter, the concentrations corresponding to a Donnan equilibrium are not those for which the regulating functions for the two end solutions become identical nor do they insure the absence from the system of unwanted moving concentration boundaries. In the electrophoresis of presumably pure proteins in acid media, where the hydrogen ion can no longer be neglected as a constituent and where extensive binding of small ions and molecules is thought to occur, patterns are frequently obtained that have not been satisfactorily explained (50). It would be of interest to know to what extent the patterns are a result of the preparation of the end solutions by dialysis.

VI. MEASUREMENT OF CONDUCTIVITY

A. The Conductivity Cell

Conductances are determined with the aid of the shielded bridge and conductivity cell developed by Shedlovsky (51). As shown in Fig. 15 the electrodes of the cell are hollow truncated platinum cones *e-e*, the outer surfaces of which are sealed to the glass and make contact with mercury in the tubes *t-t* by means of platinum wires. The inner surfaces that make contact with the solution are platinized. Owing to the relatively unimpeded flow when the cell is filled through tubes *f-f*, there is little tendency for air bubbles to be trapped, an advantage in the case of protein solutions. The glass rod *r-r* supports the cell in a half-gallon Thermos vessel No. 8621 that serves, when filled with crushed ice and distilled water, as a thermostat for the conductance measurement.

A cell of this type requires 3 ml. of solution and has a constant, K, in the range from 10 to 20. This constant is determined with the aid of a solution of known conductance, e.g. one containing 7.4788 gm. of potassium chloride per kilogram of water (values against brass weights in air) has a specific conductance at 0°C. of 0.0071379 mho. If the resistance of the cell when filled with this solution is R' ohms, then $K = 0.0071379\ R'$. Now, if another solution has a resistance of R ohms, its specific conductance is $\kappa = K/R$. In order to conserve protein the dried cell is filled with this solution without rinsing.

A cell having a constant of 10 to 20 has a resistance of a few thousand ohms when filled with most of the 0.1 M buffers. In this range the conductivity bridge described below is most sensitive. For dilute buffer solutions a cell having a constant of 1 to 2 may be used or that shown in Fig. 15 may be shunted with a resistance having an accurately determined value R_1 of, say, 10,000 ohms. If R_2 is now the balancing resistance, that of the cell is $R = R_1R_2/(R_1 - R_2)$.

B. The Conductivity Bridge

A sensitive and precise conductivity bridge is obtained by providing the Leeds and Northrup ratio box number 1153 and their four-dial decade resistance box number 4747 with an oscillator, an amplifier, and head-

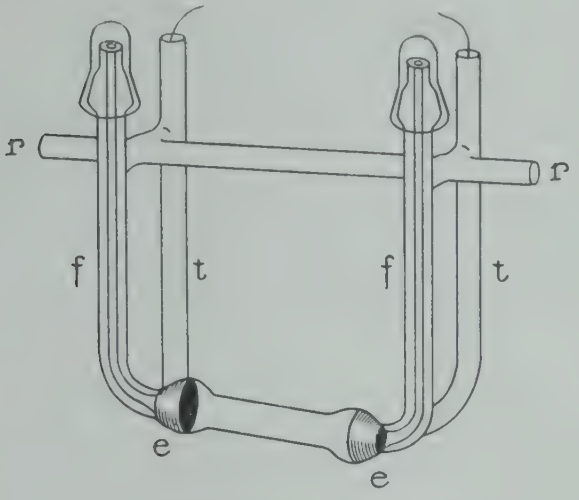

FIG. 15. Shedlovsky conductivity cell.

phones. An input frequency of about 2000 cycles is a convenient one, and Shedlovsky (51) has described suitable oscillators and amplifiers for this bridge. Since the ratio arms of the Wheatstone bridge are fixed at 1000 ohms each, the cell resistance, when balanced, is given directly to the nearest ohm by the setting of the decade dials and to the nearest 0.01 ohm by the graduated 1.1 ohm rheostat in the ratio box.

C. Conductivity Changes during Dialysis

Although the conductance of the buffer solution against which the protein has been dialyzed is not used in mobility computations, a comparison of its conductance with that of the protein solution usually indicates the effectiveness of the dialysis. As the Donnan equilibrium is attained the difference between the conductances of the two solutions approaches a small but constant value. The term equilibrium is used here in the restricted sense that the diffusible solutes are in equilibrium across the semipermeable membrane but not the water. It is seldom that the dialysis bag containing the protein is closed in such a manner that sufficient "turgor" pressure is developed to prevent the very slow entrance of water after the diffusible solute equilibrium is essentially complete.

VII. CONTROL OF CONVECTION

A. Sources of Convection

By convection is meant the circulation of solution in the channel resulting from (a) the horizontal density gradients accompanying the dissipation of heat generated by the passage of current, i.e. the Joule heat, (b) electroosmotic streaming due to the net charge in the liquid that neutralizes the fixed charge on the walls of the channel, and (*c*) the development, by electrophoresis, of layers of solution less dense than those above or more dense than those underneath. Convection due to (*c*) arises, for example, if the value of the regulating function for the protein solution is less than that for the buffer and will be called Kohlrausch convection. Preparation of the protein solution by dialysis generally avoids this. The β-globulin disturbance may be an example of (*c*). This is the abnormally sharp spike in the descending β-globulin boundary (Fig. 12) that is produced by localized convection.

B. The Low-Temperature Thermostat

In electrophoresis in the Tiselius cell it may be presumed that some circulation always occurs in the conducting, homogeneous columns of solution between boundaries. The question then arises as to the magnitude of the vertical density gradient in a boundary that is required to prevent the "erosion" of its edges by this circulation. In the case of the Joule heat this is generated approximately uniformly in each volume element of the conducting liquid but is dissipated to the thermostat fluid through the channel walls. In the narrow channel of the Tiselius cell most of this energy flow is through the 25 × 86-mm. side walls (Fig. 1). Consequently the liquid at the center of the channel is hotter than at the wall. At room temperature the density decreases with rising temperature, thus causing the liquid to rise in the center and return along the walls. At 4°C., however, the density of water is independent of temperature and the horizontal temperature gradient, although much the same as at higher thermostat temperatures, now produces very little circulation. The use of the low-temperature thermostat by Tiselius, together with the narrow channel of rectangular cross section, also introduced by him (1), represents an outstanding contribution in moving boundary work. Mooney (52) has computed the complex pattern of flow that should result when the temperature drop between the center of the channel and the wall, which is assumed to be independent of the resulting flow, includes the temperature of maximum density.

The optimum thermostat setting is not 4°, however, for two reasons. First, allowance must be made for the fact that the mean temperature of the conducting solution is above that of the thermostat. Second, and most important, practically all solutes lower the temperature of maximum density of water. For 0.1 *M* sodium acetate this temperature is 2.85° and would be still lower for an acetate buffer of this ionic strength. For the solutions most commonly used in free electrophoresis a thermostat setting of 1°C. is satisfactory.

C. Joule Convection and the Density-Temperature Function

The correlation between disturbances due to the Joule heat and the slope of the density-temperature curve is close. Moore (53) found, for example, that the disturbances at the serum-protein boundaries in a urea-rich buffer at 0° were not unlike those in a urea-free buffer at 17° where the slope of the density-temperature curve for water was similar to that of the aqueous urea at the lower temperature. The convection caused the pattern of each boundary to split into several sharp spikes whose number and shape changed erratically with time.

The maximum power, $P = I^2/s^2\kappa$, that can be dissipated from the standard cell, where $s = 0.75$ cm.^{2}, without serious convective disturbances appears to be about 0.15 watts per cubic centimeter. For 0.1 M sodium acetate $\kappa = 0.0038$, and this corresponds to a maximum current, I, of 18 milliamperes or a maximum field strength, E, of 6.3 volts per centimeter. In the course of their study of plant globulins that were sufficiently soluble only at temperatures above 1°, Johnson and Shooter (54) found experimentally that at 20° the field strength had to be decreased to one-third or less of the permissible value at the low temperature.

D. Electroosmotic Convection

Electroosmosis also contributes to the circulation in a homogeneous conducting liquid. Whereas the flow pattern resulting from the dissipation of the Joule heat is independent of the direction of the current relative to gravity, that due to electroosmosis is polarized. Thus if the two effects combine to increase the circulation in one side of the channel some compensation will occur in the other side.

E. Nonplanar Boundaries

The erosion of a boundary by the circulation of fluid on either side gives rise to what Trautman and Gofman (55) have termed nonplanar boundaries, for the observation of which an optical system that focuses both cell coordinates is essential, e.g. the schlieren band system of Fig. 4. The edges of the band conjugate to a nonplanar boundary are not horizontal. Almost all boundaries exhibit some departure from planarity with the edge of the schlieren diaphragm close to the normal slit image, but if this distortion persists as the diaphragm is lowered and the band narrows, excessive convection is indicated.

In the free electrophoresis of vaccinia (56) and similar suspensions the particles contribute little to the density of the liquid and a boundary across which they disappear is thus susceptible to erosion. This frequently appears as a chimney effect. Density-gradient electrophoresis of these materials is thus indicated. Nonplanar boundaries are also encountered in the electrophoresis of substances, such as collagen and the nucleic acids, whose solutions have excessive non-Newtonian viscosity. The hydrodynamic properties of these solutions are not those of a true liquid, and progress in adapting electrophoresis to their study has been slow (57).

VIII. Electrodes and Electrode Vessels

A. Silver-Silver Chloride Electrodes

Although gassing electrodes are used both in analytical and in preparative zone electrophoresis, and in some preparative free electrophoresis (58), the nongassing silver-silver chloride electrodes are employed in the Tiselius apparatus. The reaction taking place at the cathode or negative terminal is

$$\text{AgCl} + \text{electron} \rightarrow \text{Ag} + \text{Cl}^- \qquad \text{(I)}$$

An adherent, conducting layer of silver chloride must, therefore, be present on this electrode. At the anode the reverse reaction occurs, i.e.

$$\text{Ag} + \text{Cl}^- \rightarrow \text{AgCl} + \text{electron} \qquad \text{(II)}$$

Since the silver chloride has a larger volume than the silver from which it is formed, the compact structure of crystalline silver does not favor a high capacity. Thus the electrodes, which are frequently constructed by winding a flat and a corrugated strip of sheet silver into a tight spiral, should present a relatively large surface to the chloride solution bathing them and should be "formed" as follows.

New electrodes may be cleaned and very lightly etched by momentary immersion in dilute nitric acid. After rinsing they are then made the anode and cathode, respectively, in an approximately 1 *M* solution of hydrochloric acid and a current of about 20 milliamperes passed. Hydrogen gas is liberated at the cathode and an adherent brownish-purple deposit of silver chloride forms at the anode. After a time gas, mostly chlorine, begins to be liberated at the anode. When this occurs the connections are reversed and silver chloride is deposited on what was originally the cathode until it in turn begins to gas. The connections are again reversed and another cycle begun. This is repeated until the desired capacity is attained. It will be observed that with each cycle more silver chloride is formed at the anode before gas evolution begins here than during the previous cycle. Moreover, as the deposits of "available" porous silver on the one electrode and of silver chloride on the other are thus built up, heavier currents can be carried without loss of active material. The currents should never be of such magnitude, however, as to produce blisters. If not abused in service, properly formed electrodes last almost indefinitely.

B. Foreign Ions

The ions involved in the electrode reactions frequently differ from those in the moving boundary channel, and by interposing a large volume of buffer it is possible to keep these foreign ions from penetrating into that channel. Such penetration alters the electric fields in the boundary system and can be detected analytically or, more conveniently, by observing a change in the boundary displacement per unit quantity of electricity passed. If the transport of foreign ions is convection-free the following relation, suggested by Tiselius (1), is valid.

$$V = \frac{|u_R|\, v}{|u_A - u_B|} \tag{2}$$

Here V is the volume of buffer between the chloride solution at the electrode and the channel that will just keep the foreign ion of highest mobility, $|u_R|$, out of the channel as the boundaries due to the proteins A and B become separated by the volume v.

C. Kohlrausch Convection in the Electrode Vessels

It is not possible, however, to design electrode vessels that provide convection-free transport with all buffers. Some of the problems encountered may be illustrated by considering the boundary systems that develop, on passage of the current, at the junction of the 1 C NaCl buffer solution just above an electrode. Letting NaS represent the buffer salt, and considering the buffer acid HS as an electrically neutral component of a mixed solvent, the boundary system at the cathode is

(cathode) $\mathrm{NaS}(c) \leftarrow \mathrm{NaCl}(b) :: \mathrm{NaCl}(a)$

whereas at the anode it is

(anode) $\mathrm{NaCl}(\alpha) \leftarrow \mathrm{NaS}(\beta) :: \mathrm{NaS}(\gamma)$

If S is the acetate ion, a slow ion follows a fast one at the $\alpha\beta$ boundary descending into the strong chloride solution α and this boundary is sharp. Moreover, the density of the β solution is intermediate between that of α and γ and the transport is free of Kohlrausch convection. The ion, sodium, migrating into the overlying buffer is not a foreign species and thus quite a small volume of buffer interposed between the anolyte and the channel would be adequate.

At the rising boundary bc a fast ion, chloride, follows, or rather overtakes, a slow one, acetate, and this boundary spreads very rapidly indeed, with the velocity of the chloride ions in the leading edge of this boundary being determined by the field strength, E_c, in the overlying buffer c. This is the situation envisaged by Tiselius that leads directly to equation (2). Owing to the spread of the bc boundary the solution b occupies a relatively small volume, but its density is intermediate between the densities of a and c and this boundary system is also free of Kohlrausch convection.

Brattsten's observations (59) of these acetate-chloride boundary systems in the channel of the Tiselius cell are in accord with the known mobility and density relationships described above. However, when some other buffers were examined in this manner, she noted convection below the concentration boundary in the anode side, and above that boundary in the cathode limb, as though the current was trying to generate a β solution of greater density than the underlying chloride and a b solution of less density than the overlying buffer. This is the expected behavior if the S ion makes a sufficiently large contribution to the density of the solution and, as suggested earlier in this chapter, is called Kohlrausch convection. Since this convection in the cathode vessel may carry chloride ion to the level of the side arm more quickly than by migration alone, V in equation (2) is a minimum value.

Since the number of equivalents of chloride ion in the solution bathing an electrode greatly exceeds the number required for the electrode reaction, the concentration changes here are small. The concentrated solution of sodium chloride appearing at the cathode as a result of reaction (1) and the downward migration of sodium ion falls to the bottom of the electrode cup to form a dense layer. At the anode the disappearance of this quantity of sodium chloride reduces the concentration below the $\alpha\beta$ boundary.

D. Prolonged Electrophoresis

In experiments requiring the passage of a large number of coulombs several devices are used, either singly or in combination, to prevent the appearance of foreign ions in the boundary channel. One of the simplest is to interpose a layer, c, of concentrated buffer (60) between the chloride solution and the buffer, d, against which the protein solution has been dialyzed. If no convection occurs, the effective volume of the vessel is thereby increased by an amount, $V^c(C^c - C^d)/C^d$, whereas any Kohlrausch convection is largely restricted to the layer of concentrated buffer. Since a gravitationally unstable rising boundary is stable as a descending boundary, and conversely, another effective device is to require the ions to migrate both with and against gravity in their transit from the electrode region to the channel. This is usually accomplished (61, 62) by providing the electrode vessel with an inner tube in which the ions move, followed by migration in the opposite direction in the annulus between the tube and vessel before reaching the channel. For example, if chloride ions are carried, by Kohlrausch convection, say, through a diethylbarbiturate buffer to the level of the side arms in Fig. 2, on entering the tube above the channel a gravitationally stable, though rapidly spreading, boundary would form.

Little is known, however, about the role of convection in the electrode vessels actually employed. The work of Gordon and Kay (63) suggests that the flow velocity resulting from positive density gradients is often of the same magnitude, or even less, than the velocity of ion migration. The tendency has been to make the vessels of much larger volume than equation (2) requires.

IX. Preparative Free Electrophoresis

A. Recovery of Samples

Many naturally occurring protein mixtures possess one or more activities of biological interest, and it is frequently desirable to recover the electrophoretically separated components for further study. The procedure may be described with the aid of Fig. 16, b, which is an elaboration of Fig. 1 of the preceding chapter. Since the left-hand side of the channel is open to the atmosphere a thin-walled glass capillary, attached to the empty syringe of the compensator with small-bore tubing, is lowered until its tip is just above the boundary at h_B. With the compensator in reverse the solution of pure A is withdrawn until the boundary at h_A falls to the

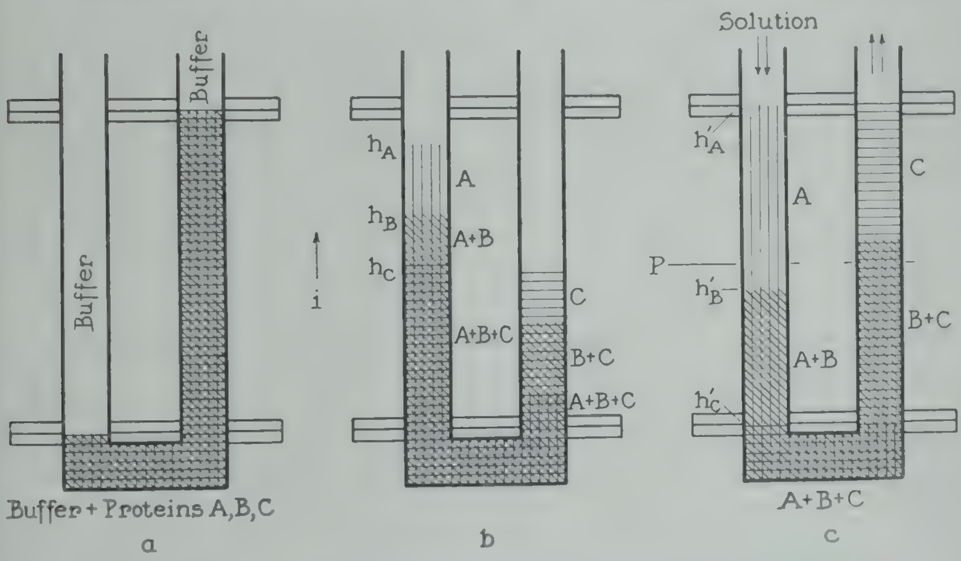

Fig. 16. Ideal electrophoresis of a protein mixture.

level of the tip, the entire operation being monitored with the aid of the schlieren camera. Similarly, a mixture of A and B would be obtained by inserting the tip to the level h_c and withdrawing the solution between this boundary and the one at h_B. In the case of the separated layers in the right-hand channel, access to these is had on removal of the stopper p (Fig. 2) after immobilization of the channel contents by isolation of the bottom section of the cell.

As is evident from Fig. 16, b, the component B of intermediate mobility is mixed with A in one side and with C in the other side. Either of these solutions can be recovered and used for the separation of B in a second experiment or a portion of this component may be recovered more directly as follows (64). After removal of the layer between h_A and h_B the uppermost boundary in the left-hand channel is between buffer and a mixture of A and B. If current is now passed in a direction opposite to that used in the original separation, B lags behind A as the boundaries now descend and a layer of pure B is thus formed. Similarly, if the layer of pure C has also been removed, B could be recovered from the right-hand channel following reversal of the current.

A glance at actual patterns of protein mixtures indicates that Fig. 16 represents an idealized process. The spreading of real boundaries as they move and the departures from enantiography both tend to reduce the quantity of pure material that can be recovered. A low ionic strength frequently favors separation of the most mobile component since protein mobilities increase in magnitude with decreasing salt concentration and the rising boundaries are generally sharp. The accompanying spreading of the descending boundaries makes recovery of the slowest component difficult, however.

B. Countercurrent Electrophoresis

It is also evident from Fig. 16, b that if electrophoresis is continued as indicated, boundaries will migrate into the top and bottom sections, respectively, before any large proportions of A and C have been separated. Movement of a boundary into the bottom section always results in convection and is to be avoided. If, however, the slowest boundary is given an apparent velocity of zero, as is indicated in Fig. 16, c, while the leading boundary moves through the height of the channel, much larger proportions of A and C can be separated. A boundary can be given any apparent velocity desired by injection, or withdrawal, at the proper rate, of buffer into the closed side of the apparatus with the aid of the compensator. The movement of the boundaries against a flow of solution in the opposite direction, whereby they are kept in the cell channel, is termed countercurrent electrophoresis. It has the effect of extending indefinitely the height of the channel and is used not only in preparative work but also in the resolution of protein ions of slightly differing mobilities (65). In countercurrent electrophoresis the essentially stationary concentration boundaries present a problem since one is carried by the counterflow into the top section while the other is carried into the bottom section, thereby initiating convection. As was described in the previous chapter these boundaries can be eliminated by proper dilution of the protein solution following dialysis (66). Modifications of the Tiselius apparatus have been described that permit continuous removal of a fraction as it is separated electrophoretically (67).

X. Conclusion

In spite of its limitations and the complexity of the equipment, free electrophoresis remains the mildest of the procedures for the analysis of protein mixtures and for the preparation of purified proteins. It also provides for the most precise electrochemical characterization of proteins. Throughout the process of extraction, dialysis, and electrophoresis the temperature may be maintained near 0°C. The electric fields used in electrophoresis are quite negligible in comparison with the local electric fields of the protein ions and are thus without harmful effect. At no stage in the process do the proteins come into contact with nonaqueous phases, or large areas of a disperse solid phase, at which irreversible changes can occur. The failure of free electrophoresis to permit complete separation of the components of a mixture becomes an advantage in the case of many protein-protein, protein-small ion, and protein-small molecule interactions where the departure of the patterns from enantiography affords clues to the interactions.

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Chapter 5

Paper Electrophoresis

CH. WUNDERLY

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I. INTRODUCTION

A. Historical Development

The first indication for the use of "paper" as a supporting medium was given by König (1); in 1939, together with von Klobusitzky (2), he reported using electrophoresis on paper strips saturated with an electrolyte solution to isolate a yellow pigment from snake venom and also to separate dye mixtures. Therefore, paper electrophoresis is several years older than the modern form of paper chromatography, which stems from the publication of "Quantitative Analysis of Proteins: A Partition Chromatographic Method Using Paper" in 1944 by Consden *et al.* (3). It is chronologically important to mention this fact since the studies of the later years indicate a tendency to combine the technique of chromatography with paper electrophoresis. Thus, almost simultaneous reports on two-dimensional paper chromatography of amino acid and peptide mixtures with the aid of direct current were made in 1948 by Haugaard and Kroner (4), Wieland (5), and Wieland and Fischer (6) and, in 1950, by Biserte (7). In 1949, there appeared significant preliminary investigations (8,9), and by 1950 the value of paper electrophoresis for the separation of mixtures of macromolecular substances had been demonstrated unequivocally in the publications of Cremer and Tiselius (10), Durrum (11), Kraus and Smith (12), McDonald *et al.* (13), Turba and Enenkel (14), and Grassmann and Hannig (15), most of which were concerned with the separation of serum proteins. The succeeding years witnessed a flood of studies which in part proposed improvements and simplifications and in part also defined the scope of the methods. The way was clear for the development of paper electrophoresis, which promises to be as useful and versatile for macromolecular substances as paper chromatography has proved to be for materials of smaller molecular weight.

B. Terminology

Among all the porous supporting media which have been proposed for the stabilization of the electrolyte solution, filter paper has found the widest distribution. In view of the many preliminary studies mentioned above, its use was in the wind and it is not surprising that in 1950 there appeared six procedures for paper electrophoresis which were developed independently of each other and without knowledge of the method of P. König (16). Various authors have employed different names for the method.

The earliest studies of the transport of dissolved substances by electric current are those of the physicists Hittorf in 1953 and Oliver Lodge in 1866, but it was Hardy (17), the founder of colloid chemistry, who coined the term "cataphoresis" in 1899. Then in 1909, L. Michaelis (18) named the migration of colloid ions in the electric field "electrophoresis." As examples of the period of transition of paper chromatography to paper electrophoresis, we may cite the work of Haugaard and Kroner (4), entitled "Partition Chromatography of Amino Acids with Applied Voltage." Cremer and Tiselius (10) as well as Turba and Enenkel (14) called the process "electrophoresis of protein in filter paper"; Durrum (11) called it "a microelectrophoretic and microionophoretic technique"; McDonald and associates (13) proposed "ionography"; the corresponding terms, "ionograph" and "ionogram", have been employed (19). In the succeeding years, "electrochromatography" (20), "zone electrophoresis" (21), "electrochromatophoresis" and "electromigration" (22) were proposed. In looking through the titles of the pertinent publications which have appeared up to the present, it is seen that the term "*Papierelektrophorese*" predominates clearly in Sweden, Germany, and Switzerland; the analogous expressions are "*elettroforesi su carta*" in Italy, "*microélectrophorèse sur papier*" in France, "*electroforesis en papel*" in Spain, and "*electroforesa na papire*" in Czechoslovakia. In the Anglo-Saxon literature, on the other hand, the term "zone electrophoresis" is used frequently.

It may be added that in the expression "paper electrophoresis" the material nature of the supporting medium is already stated, whereas zones of separated substances appear as well in starch or agar, so that it would be better to say "zone electrophoresis in paper." "Ionography" and "electromigration" are applicable generally to electromovements in stabilized electrolytes. It has become common usage to define ionophoresis primarily as the electromovement of ions and electrophoresis as that of colloid particles. This distinction is arbitrary since dissociable colloids migrate as ions of colloidal dimensions; or the colloid may adsorb ions from the environment on its surface and thus participate indirectly in conduction. The migration velocity of colloid particles in free electrophoresis is of the same order of magnitude as that of common ions. The inhibitory influence of the colloidal dimension is approximately compensated for by the number of charges of the colloid particles.

The terms "electrochromatography" and "electrochromatophoresis" designate procedures (20, 23–26) in which a chromatographic component is included; among these, there are also arrangements for the continuous separation of mixtures on filter paper (cf. Section III, A, 3). Two-dimensional paper electrophoresis (27) has also been proposed, as well as the term "electrorheophoresis" (28), for procedures in which the evaporation of the fluid is not prevented but controlled. The original method of electrophoresis according to Tiselius-Philpott-Svensson is called the free or classical form; the term "ancestral" (29) is an indication of the rapid change in significance and application of modern analytical methods.

II. THEORETICAL BASIS

A. Migration in a Solid Supporting Medium

If electrophoretic migration is allowed to proceed within filter paper or other porous media, the technical advantage over free electrophoresis lies in the stabilization of the migration. The latter is fixed, in a way, and cannot be influenced by heat-caused convection currents as in the always labile free electrophoresis. Consequently, the equipment can be made much simpler.

Migration in paper depends, first, on the properties of the ion, the magnitude and sign of its net charge, on its colloidally dispersed (protein ion) or molecularly dispersed (amino acid, pigment, etc.) nature, on its spherical or threadlike form; further, it depends on physicochemical quantities such as electrolyte concentration, ionic strength, hydrogen ion concentration, temperature, and viscosity. Besides electrical entities such as current density and field intensity, the texture of the filter paper is also important, since the latter, as the solid supporting medium, offers the electromigration a certain resistance. The adsorption on the cellulose depends as much on chemical as on physical factors; in this regard, the starting materials in manufacture as well as the degree of purity of the cellulose play a significant role. If the cellulose is first washed in acid or if ionizable groups are introduced by appropriate pretreatment, the sorption relationships again change completely. From what has been said it follows that a theoretical treatment of electromigration in cellulose as a capillary system is difficult. Kunkel and Tiselius (30) were the first to point out how the two expressions for the field intensity in free electrophoresis,

$$\frac{V}{l} \quad \text{and} \quad \frac{i}{qk}$$

(where V = volts, l = length of the channel, i = current density in amperes, q = cross section of the U-tube in cm.^{2}, k = conductivity in ohms^{-1} cm.$^{-1}$), take on different values in paper electrophoresis. They consider the reason for this difference to lie in the frequently sinuous path followed by the protein molecule in the filter paper; this path does not correspond directly to the shortest distance, d, but is greater by the factor l'/l. This factor depends on the brand of filter paper and therefore assumes the character of a material constant. Whereas in free electrophoresis the path traversed by a protein molecule is given by the expression $d = uti/qk$, the term for paper electrophoresis is $d' = uti/q_ak$, where $d' = d \times l'/l$, and q_a is the cross-sectional area of the paper strip.

Kunkel and Tiselius (30) observed that the measured mobility u_a of serum albumin depends on the paper employed, but that all values become approximately identical after multiplication with the correction factor l/l'. A comparison of the corrected mobilities for all serum fractions on Munktell paper 20/150 with those of Dole (31) obtained in free electrophoresis, gave the following results ($u \times 10^5$): for albumin the values were, respectively, 6.48 and 5.94; for α_1-globulin, 5.15 and 5.07; for α_2-globulin, 3.97 and 4.08; for β-globulin, 3.02 and 2.83; and finally for γ-globulin, 1.27 and 1.02. The measurements of McDonald and Spitzer (32) demonstrated the influence of the molecular volume on the mobility in filter paper; in Munktell paper 20/150, with a field intensity of 5 volts/cm. and at pH 8.3, they found mobilities μ/sec./volt/cm. of 1.31 for nickel ion, 0.90 for aspartic acid, 0.32 for polyethylene glycol, and 0.30 for polyvinylpyrrolidone. The migration of small ions is less influenced by the type of paper than that of large, colloidally dispersed ions. A more detailed analysis of these and other correction factors is to be found in the following chapter of this book.

B. Dependence of Mobility on Time and Field Intensity

As a consequence of the progressive imbibition of the paper strip with buffer solution, there occurs a small increase in conductivity during the first 20 minutes of paper electrophoresis. During the succeeding hours, the field intensity, current density, and pH value remain practically constant at 10°C. as well as at 25°C. (33); this is important because the migration velocity of a component of the mixture to be separated depends primarily on the factors mentioned above. The current intensity to be applied has an upper limit by the Joule heat developed in the paper; on it depends the evaporation and the enrichment in buffer ions caused by it. But the ionic mobilities and free diffusion also increase with increasing temperature of the paper strip. The heat (H) evolved, in calories per second, is given by the quotient

$$H = \frac{RI^2}{4.18} = \frac{VI}{4.18}$$

where I is the current intensity, V the potential difference, and R the resistance of the paper strip. In order to obtain a better dissipation of the produced heat, the use of a helium atmosphere within the paper electrophoresis apparatus has been proposed (34). Because of its low atomic weight, the heat conductivity of helium at 0°C. is about six times that of air. Its use was suggested whenever the electrical energy per square centimeter surface of the paper exceeds 0.005 watts (at 25°C.). If a high applied terminal voltage is intentionally selected (350 volts) in order to decrease the duration of paper electrophoresis (2 hours), then cooling is necessary if denaturation of the protein is to be prevented. Table I provides a summary of the mobility of serum protein under various experimental conditions.

C. Dependence of Mobility on the pH and Ionic Strength of the Buffer System

If one disregards the factors discussed above which contribute to the determination of the mobility, u, of a protein molecule in a porous sup-

TABLE I

MOBILITY OF SERUM PROTEIN UNDER VARIOUS EXPERIMENTAL CONDITIONS

Reference	Type of paper (Whatman no.)	Dimensions of paper strip (cm.)	Potential difference (volts)	Current intensity (ma)	Duration (hours)	Length of the longest path (cm.)
Durrum *et al.* (35)	2	1 × 32	220–230	0.5	3	About 3.5
Macheboeuf *et al.* (36)	1 and 2	6 × 32	320–340	3	6	16–18
Flynn and de Mayo (37)	1 and 3MM	6 × 32	120	1.5	20	16–18
Grassmann and Hannig (33)	1	4 × 40	110	4	14	9–10
Sonnet and Rodhain (38)	1	4 × 36	120	0.8–0.9	15–17	6–8
Wunderly (39)	1	7 × 30	165	6–7	8	8–9

porting medium, then the mobility obeys the expression

$$u = \frac{Qd}{4\pi r^2 \eta}$$

where Q is the charge of the protein molecule, d the distance of the electrical double layer from the protein molecule, r the radius of the molecule, and η the viscosity of the solution. The radius of the molecule can be derived from the experimentally determined diffusion constant D according to the equation

$$r = \frac{KT}{6\pi\eta D}$$

Since the radius of the molecule is squared in the preceding equation, an increase in molecular size significantly diminishes the mobility u; but if this increase in volume is also connected with anisotropy of shape, then the viscosity η also increases at the same time. This correlation is involved in determining the failure of attempts to separate blood plasma in paper electrophoresis; fibrinogen with a molecular weight of 300,000 to 400,000 exhibits so low a mobility in paper that it partially remains behind in place of γ-globulin and thus falsifies the determination of the latter (11, 16, 37, 41). Even the determination of fibrinogen as the difference between parallel electrophoretic runs of the serum and the plasma is not satisfactory in all instances (cf. 42). On the other hand, paper electrophoresis offers an excellent method by which even slight morphological alterations of the protein molecules, as they may occur as a consequence of denaturation, can be made measurable (cf. 43, 44).

Extremely large protein molecules, e.g. macroglobulin (molecular weight 1,000,000), with correspondingly slow diffusion exhibit sharply defined boundaries in paper electrophoresis, whereas albumin (molecular weight 69,000) usually has a sharp front boundary but sometimes an indistinct back boundary.

On the basis of the pH which is selected, the mobility, u, of the protein molecule may be influenced by the change of the charge, Q, whereas with variations of the ionic strength of the buffer system the distance, d, of the ionic double layer is altered. If the ionic strength increases the distance d from the surface of the molecule becomes progressively smaller and at the same time the electrokinetic (zeta) potential ζ drops off exponentially. This zeta potential, which forms on the surface of the layer of solution which surrounds the protein molecule, is related to the ionic strength μ at 25°C. in the following way:

$$\zeta = \frac{4\pi\sigma}{D} \frac{1}{0.33 \times 10^8 \sqrt{\mu}}$$

In this equation, σ is the charge density on the surface of the protein molecule and D the dielectric constant. The zeta potential is in direct relation with electroosmotic mobility u

$$u = \zeta D / 4\pi\eta$$

where η is the viscosity of the medium.

Since amino acids, peptides, and proteins are ampholytes, the pH of the buffer system exerts great influence on their mobility. If the pH of the buffer lies on the acid side of the isoelectric point, the ampholyte bears a positive charge and migrates toward the cathode; if, on the other hand, the pH is on the alkaline side of the isoelectric point, the ampholyte carries a negative net charge and migrates to the anode. Figure 1 gives the relationships for glutamic acid (45). McDonald and Williamson (46) reported on an arrangement where the electrophoresis takes place in the paper strip in a pH gradient. According to Kolin (47), the process is called isoelectric separation.

However, the chemical composition of the buffer system is also of importance for the result of paper electrophoresis. Since the buffer ions are adsorbed on the protein molecule, they contribute to its charge; the influence of the buffer ions is not purely quantitative, but the ionic valence also plays a role.

In paper electrophoresis, buffer systems with diethylbarbituric acid

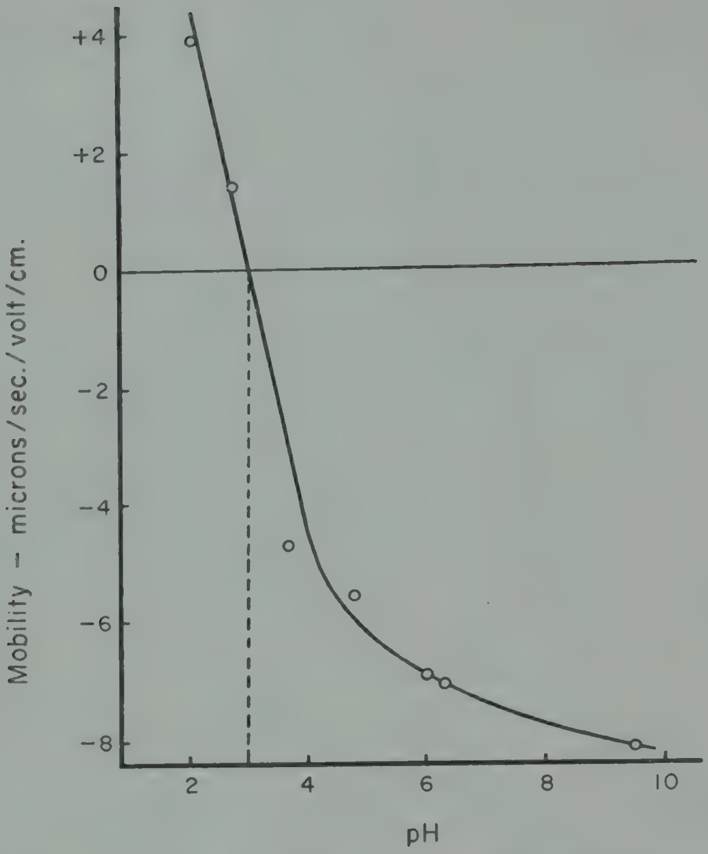

FIG. 1. Mobility of glutamic acid as a function of the pH of the buffer solution. The isoelectric point is at pH 3.1. Cf. (45).

(pk = 7.43) at pH 8.6 have been used most frequently; they guarantee a sufficiently large buffer capacity with low ionic strength and thus provide the prerequisite for adequate migration of the ions to be separated. The buffer systems (48–50) listed in Table II can be recommended. The buffer

TABLE II

DIETHYLBARBITURATE BUFFER SYSTEMS

Composition	Total quantity (liters)	pH	Ionic strength
10.3 gm. Na diethylbarbiturate and 1.84 gm. diethylbarbituric acid	1	8.6	0.06
17.1 gm. Na diethylbarbiturate and 3.12 gm. diethylbarbituric acid	1	8.6	0.083
29.34 gm. Na diethylbarbiturate 19.42 gm. Na acetate, and 180 ml. 0.1 *N* HCl	3	8.6	0.10

solutions may be used four or five times if the poles are reversed each time.

If paper electrophoresis is to be carried out at pH 6.4, the buffer of choice is 0.033 *M* Sörensen phosphate buffer; at pH 3.7, 0.1 *M* sodium acetate-acetic acid buffer is used. For paper electrophoresis of sugars and sugar derivatives, borate buffer (51, 52) at pH 9.2 is suitable (0.2 *M* boric acid and 0.2 *M* KOH, 1:1). A borate buffer of pH 8.6 contains 8.80 gm. sodium borate and 4.65 gm. boric acid per liter; in the separation of serum proteins it sometimes permits a distinction between β_1- and β_2-globulin, whereas a satisfactory separation of α_1- and α_2-globulin has not been achieved.

If it is desired to determine the ultraviolet absorption of eluted protein fractions, it is advantageous to employ an alkaline glycine buffer of pH 8.8 and $\mu = 0.07$ (39); in contrast to the diethylbarbituric acid buffer systems, glycine does not exhibit any absorption of its own in the absorption range of the proteins. This type of measurement permits a determination of unchanged proteins rather than the staining of their heat-denatured products. For summaries on the composition of buffer systems, reference may be made to the papers of Miller and Golder (53) and of McFarren (54).

D. Electroosmosis and Evaporation

In addition to field intensity and current density, whose effect on the migrating materials has been described above, the materials are also affected by an electroosmotic force pointing in the opposite direction. The concomitant processes are designated as electrokinetic phenomena. Electroosmosis is due primarily to the negative charge which the fiber surface of the filter paper acquires with respect to the buffer fluid; besides the zeta potential, the viscosity of the solution and the nature of the cellulose are also determinants. Since the filter paper as a capillary system represents the stationary phase, the effect is transferred to the buffer solution; the latter is positively charged with respect to the paper and therefore exhibits a flow toward the cathode. This flow is approximately proportional to the potential gradient and can be measured with substances which do not participate in conduction and are not adsorbed on cellulose. For this purpose, caffeine (55), xylose and glucose (56), formaldehyde, H_2O_2, thioacetamide, allylthiourea, hydroquinone, dextran (30), and radioactive sucrose (57), as well as creatinine (58) and *o*-nitroaniline (59) have been proposed. By determining their mobility, a correction value is obtained which can be added to the mobility of the anions and subtracted from that of the cations. The molecular volume of the yellow *o*-nitroaniline corresponds approximately to that of the amino acids, while dextran can be obtained in orders of magnitude which approach the molecular weight of albumin or of the globulins.

The fluid movement within the paper strip is also influenced by the following "suction effects." A "siphon effect" may occur at uneven levels of the buffer solutions; a connection of the two buffer solutions by a tube facilitates the equalization of levels. Of greater importance is the evaporation of fluid as a consequence of the heat evolution caused by the passage of current. The latter increases with the square of the field intensity if the conductivity in the paper strip remains constant; it leads to a concentration of the buffer solution in the strip and thereby alters the ionic strength. If the paper electrophoresis is carried out in a set-up where the paper strip is suspended over an edge, then evaporation is strongest in those portions of the strip which are nearest to the buffer solution. As shown by Macheboeuf and associates (60, 61), the evaporation gradually decreases toward the center of the paper strip; it was possible to characterize this passive fluid movement by means of drops of a glucose solution applied at different spots. After 6 hours, all the drops of glucose are found at the same level as a result of the equilibrium state. Similar effects were achieved with serum proteins and with rapidly diffusing inorganic ions. By controlling the evaporation (lateral openings in the moist chamber), the fluid movement can be regulated so that the final location of an ion becomes independent of its origin. Macheboeuf designated this technique "electrorheophoresis."

For the measurement of the migration velocity of ions, the field intensity should remain as constant as possible. This objective is favored by diminishing the evaporation through the admixture of 5–15% glycerin or propylene glycol to the buffer (62). The use of thin walls made of a good heat-conducting material for the moist chamber is advantageous for a rapid heat equilibrium with the environmental air. Condensation on the inner wall remains small and so does the formation of drops. Thus, the filter paper whose sorption relationships promote chromatographic effects the least, is the most suitable for the measurement of the migration velocity.

III. METHODS

A. Technique

The technique of paper electrophoresis exhibits a remarkable multiplicity of forms of execution. While the majority of instruments were constructed for analytical purposes, arrangements are also available for continuous separation which permit preparative runs. A number of surveys dealing with methodology may be cited: Symposium Utrecht (63), Antonini and Piva (64), Wunderly (65), Ciba Foundation Symposium (66), Peeters and Vuylsteke (67), Fisher (68), Kirk and Duggan (69), and the appropriate chapters in the monographs of Turba (70), Cramer (71), Block *et al.* (62), Wunderly (72), McDonald (73), and Lederer (74).

1. *In the Moist Chamber*

At first, attempts were made to carry out paper electrophoresis at higher voltages, wherefore it became necessary to draw off efficiently the heat produced by the current passage. For this purpose, the moist paper strips were placed between glass plates in a chlorobenzene bath. This arrangement, called the sandwich technique, was employed first by Cremer and Tiselius (75) and then by Schneider (76) and Schneider and Wunderly (77). With 300 volts and 20 milliamperes, it permits a reasonably good separation within 3 to 4 hours; but, nevertheless, the separation of α_1- and α_2-globulin remained unsatisfactory. Therefore, at present low field intensities and freely suspended or horizontally placed paper strips are used predominantly.

Quite early, Durrum (8, 35, 78, 79), Flynn and de Mayo (80), Macheboeuf *et al.* (81), and Wunderly and associates (82) developed the method in which the moist paper strip hangs freely over an edge. These set-ups were still constructed with simple laboratory tools; this was, so to speak, the heroic era of paper electrophoresis. The practical experiences then led to instruments which were manufactured in large series and became commercially available. Among these, we should like to emphasize the Spinco Model R*, which has been used by the author. The model was developed according to data of Williams and co-workers (83); by means of ingenious automatization, several sources of error were eliminated and thus simple, and at the same time highly reproducible, performance was assured. As shown in Fig. 2, a series of eight strips of Whatman paper 3MM, 3 × 30 cm., are placed on a detachable folding Plexiglas rack. The strips are supplied ready-cut and have small round openings at both ends through which they are attached in the dry state to the pegs on the rack. When the rack is folded and set into the rack support, the ends of the paper strips are pressed to the paper wicks placed below them. The latter serve to provide an even supply of the buffer solution and thus to prevent distortions of the protein fronts. Subdivisions of the Plexiglas trough (baffle system) diminish the diffusion of the products of electrolysis from the platinum electrodes. After the Plexiglas cover has been put into place, the system is closed except for the loading slot in the top. Through this slot, the buffer solution is distributed evenly over the papers and the longitudinal slot is closed with adhesive tape. After about a half hour, equilibrium has been established. At that time, 0.008 ml. of serum per paper strip is added with an applicator through the opened longitudinal slot. After closing the longitudinal slot, a constant current intensity of 15 milliamperes (maximum current variation ±2%) is applied using the Spinco Duostat. After 8 hours, the cover

* Spinco Division, Beckman Instruments, Inc., Belmont, California

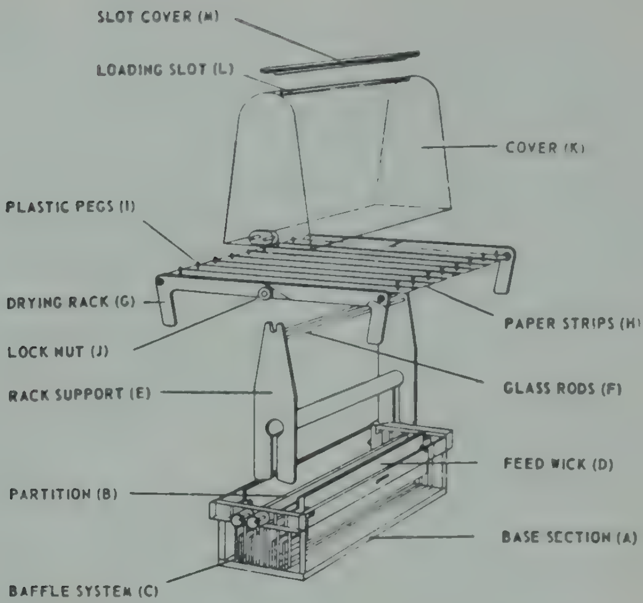

FIG. 2. The electrophoresis set-up for eight freely hanging paper strips developed by Williams *et al.* (83).

is removed and the carrying rack with the moist paper strips is unfolded and placed for 30 minutes into an oven at 110°C. The dry papers are slipped into corresponding holders made of stainless steel and dyed with bromophenol blue in closed coloring baths (cf. Section III, C, 1). After careful washing, the strips are dried again and placed singly into an "Analytrol" analyzer (cf. Section III, D, 1). The serial coloration and drying assures the procedure of remarkable uniformity of results.

Among the arrangements having paper strips in a flat, horizontal position, that described by Grassmann and co-workers (33, 84) has found the widest application. As shown in Fig. 3, the moist filter paper strip, generally Whatman No. 1 or Schleicher and Schüll No. 2043a, is placed over the flat portion of the bridge *R* in such a way that it is held in place by adhesion. In the two outer portions of the chamber, there are the platinum electrodes E_1 and E_2; they dip into a labyrinth system which provides assurance against electrolyte shifts. During the experiment the glass cover *G* is placed so that a moist chamber is formed above the space occupied by the two filter paper strips, while gaseous products of electrolysis formed in the outer portions of the chambers can escape to the outside. The two pairs of chambers are connected by small countersunk glass tubes which are filled compactly with glass wool.* If a terminal voltage of 110 volts is applied, a

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* The apparatus is sold by the firm of Bender and Hobein, Munich 15, Germany, and by Evans Electroselenium Ltd., Harlow, Essex, England. A similar arrangement is exhibited by the instruments of Shandon Scientific Co., Cromwell Place, London S.W. 7, and of Ivan Sorvall Inc., Norwalk, Connecticut.

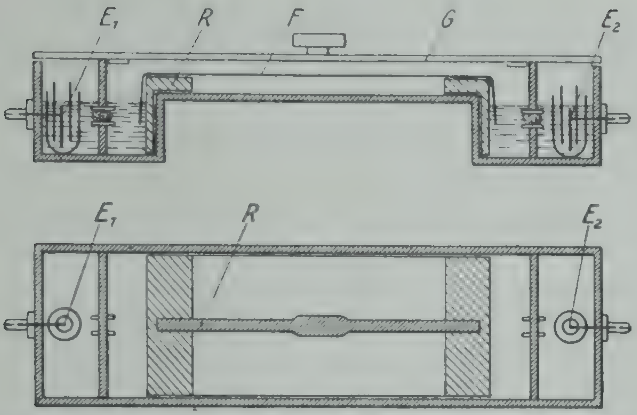

FIG. 3. Electrophoresis chamber according to Grassmann *et al.* (84). G = glass cover, E_1 and E_2 = electrodes, F = filter paper strip, R = plastic rack.

potential difference of 80 volts is established across the paper strip. The optimal amount of serum is stated to be 0.005 to 0.010 cc. insofar as the protein content of serum lies in the range of 6.5 to 8.0 gm.%. Although these instruments are of simple construction, they give entirely satisfactory results as long as full attention is devoted to the small technical details. Valmet and Svensson (85) have emphasized the importance of control determinations. The instruments developed by Franglen *et al.* (86) and by Crestfield and Allen (87) favor stable conditions in the interior and in the paper strips, thus improving precision and uniformity.

On the basis of details published by Macheboeuf and associates (60, 61), a set-up with controllable evaporation has been developed.* This can also be achieved in the apparatus designed by Dustin (88);† since the paper strips in this instance are placed on several levels, up to twenty samples of serum can be handled at the same time. A multipurpose apparatus has been realized in the "Reco E-800-2";‡ since it is equipped with a water-cooling system, potentials up to 750 volts can be applied, corresponding to a field intensity of 20 volts/cm.; the paper strips can be arranged to lie horizontally or to hang free; moreover, the use of starch gel or silica gel is possible. For the separation of amino acid mixtures, Durrum (79) evolved two-dimensional paper electrophoresis as early as 1951; the "strip transfer method" permits ionophoresis at varying pH values. Later, Mead (89) again took up this possibility for measurements in an elaborate apparatus.

In the paper mentioned above, Durrum (79), was the first to combine ascending paper chromatography with ionophoresis. The same sequence of measurements was used by Kickhöfen and Westphal (90) for amino acids and by Quastel and Van Straten (91) for serum proteins. Verhelle and Merlevede (92) first carried out one-dimensional paper chromatography with twenty-one amino acids and cut the insufficiently separated areas out of the paper; these were then subjected to paper electrophoresis. Lockhart and Abraham (93), Honegger (94), and Fisher (95) also applied this combination of paper methods successfully to their problems. If paper electrophoresis is carried out with volatile buffer systems, e.g. a combination of acetic acid and pyridine, then the dried strip can be attached to a second, larger sheet of paper and is thus ready for paper chromatography. Marini-Bettolo and collaborators (96) employed this method for the separation of alkaloids. For the isolation of the electrophoretically separable components of hirudin, Markwald (97) used a 0.2 M ammonium formate buffer of pH 4.8; for its removal, the paper strip was kept for 15 hours under high vacuum over NaOH and P_2O_5.

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‡ Research Equipment Corp., Oakland 20, California.

2. Details of the Technique

Since the technique of paper electrophoresis appears so very simple compared to that of free electrophoresis, it is sometimes overlooked that certain details must be adhered to precisely if a reliable evaluation is desired. Whereas in free electrophoresis gradients may completely disappear if small technical infractions have occurred, it happens frequently in paper electrophoresis in routine analyses that strips are evaluated whose fronts are insufficiently separated or not linear.

Serum should not be older than 24 hours since even a small decrease in dispersion of its colloidal components allows coarsely dispersed protein to remain at the origin or to cause tailing. In lipemic sera, chylomicrons are found in this spot. In both instances, this must be taken into account in the determination of the γ-globulin fraction.

If the fluid (e.g. serum, cerebrospinal fluid concentrate) is applied to the paper with a micropipette, a blunt tip should be used; in this way any roughening of the surface of the paper is avoided. If the material is to be applied over a distance longer than 2 cm., it is useful to guide the pipette along a ruler. No application should be made closer to the margin than 6–8 mm., since otherwise marginal distortions are unavoidable. After placing the material on the previously marked spot on the dry paper, the strip is allowed to dry for several minutes, then grasped at both ends and wetted with buffer fluid, starting at one end in such a way that the fluid front extends to within a few millimeters of the origin. The latter is then rapidly moistened with buffer fluid by diffusion. The moist half of the strip is gently dabbed with clean blotting paper, and then the other half of the paper strip is handled in the same way. In this way, undesirable diffusion of the applied solution is avoided and a better starting condition for the separation is provided. If pipetting is to be avoided, one may saturate a little Whatman No. 1 paper strip with serum and then place it on the origin. If it is desired not to leave the little strip in this position, one may hold it between two glass cover slips; if the longitudinal front of the strip is allowed to protrude a little, it can thus be applied to the origin of the pherogram. In order to be able to check the progress of the albumin front at any time, a cotton thread previously saturated with bromophenol blue solution is placed on the origin; staining with amido black is not influenced by this procedure.

Although the temperature coefficient of the electrophoretic separation is small, one should work under cool conditions (not in the sun) to avoid promoting evaporation. A strip of Wettex placed at the bottom of the cover prevents the annoying dripping of fluid on the paper strip. It is desirable to strive for as even a distribution of heat within the moist chamber as possible, since then the conditions in the paper are most uniform.

After completion of the paper electrophoresis, the ends of the paper which were in immediate contact with the buffer solution are first cut off with a scissors; if feeders are present, this is not necessary. Then the strips are dried for 30 minutes at 110°C. in a horizontal position. Uniform heat-denaturation is important because only denatured globulins bind bromophenol blue, in contrast to albumin.

3. Continuous Paper Electrophoresis

It is not surprising that attempts were made early to utilize paper electrophoresis also for preparative separations. For this purpose, Durrum (35) employed paper strips of 0.5–1.0 mm. thickness and thus separated 0.3 ml. of serum. Later, Wunderly (65) developed an instrument in which $4 \times 0.3 = 1.2$ ml. serum is worked up simultaneously within 16 hours at $+2°$ on 4 strips of Whatman 120 (weight: 27.7 mg./cm.^{2}) or of Munktell 20/350 (weight: 34.6 mg./cm.^{2}); the glycine buffer is at pH 8.8, $\mu = 0.07$; the yield of eluted protein fractions is about 70%; their quantitative determination is carried out by measurement of the ultraviolet absorption. Kunkel and Slater (98) utilized a package of 3–9 superimposed filter paper strips of Whatman 3MM; with 25–60 milliamperes and 100–500 volts, it is possible to separate 2–5 ml. of serum within 24 hours. Similar procedures have been used by Fischer and Behrens (99), Strain (100), and Loomeyer (101). With paper cardboard of 2 mm. thickness, as the supporting material, Hartmann and Müller (102) separated 2–3 ml. of serum. In the same way, Knedel and Althaus (103) were able to separate up to 1.0 ml. of serum on a strip (8×30 cm.) of Schleicher and Schüll paper No. 2230; 230 volts were applied and a 1:1 dilution of Michaelis buffer was used; the duration of the run was 7 hours; after a 4-hour elution with physiological saline in the refrigerator and mechanical pressing out of the strips, a high yield is achieved. The horizontal cardboard paper electrophoresis (Schleicher and Schüll No. 2230) was further improved by Schmidberger (104); it was possible to separate 50 ml. of serum or 5 gm. of a purified serum fraction within 5 hours with 300 volts and a field intensity of 9 volts/cm.; since the separation was carried out in a cold room at +6°C., the temperature in the apparatus did not rise above 26°C. The uniformity of the isolated fractions was tested with paper electrophoresis and Tiselius electrophoresis (cf. Table III).

TABLE III

COMPOSITION OF ISOLATED SERUM FRACTIONS

	Paper electrophoresis					Tiselius electrophoresis					
		Globulins					Globulins				
	Albumin	α_1	α_2	β	γ	Albumin	α_1	α_2	β_1	β_2	γ
Albumin	93.7	6.3				98.3	1.8				
α_1-Globulin	36.9	39.8	22.3			30.2	60.7	9.7			
α_2-Globulin	3.5	29.3	67.2			3.0	33.3	62.0	1.7		
β-Globulin			22.0	70.5	7.5			4.9	47.3	37.7	14.1
γ-Globulin				3.8	96.2				3.3		96.7

[a] Total β-globulin fraction.

A better degree of purity, particularly of albumin (98.6%) and γ-globulin (99.3%) was achieved by a second zone electrophoresis of the fractions; however, the purity of α_1-globulin rose only to 56.7% and that of α_2-globulin to 80.3%. These experiences indicate the limits of the procedures which are carried out in the absence of flow.

At about the same time, Grassmann and Hannig (15), using paper, and Svensson and Brattsten (105), using glass powder, described instruments in which the principle of continuous paper electrophoresis was realized (cf. 23). The former investigators later carried out a series of technical improvements; these have been included in the apparatus sold as the "Elphor V" (Bender and Hobein, Munich 15, Germany) (106). Another design is commercially available as the Spinco Model CP. The supporting medium consists of squares of extra-thick filter paper of high wet strength (Macherey and Nagel No. 819; Eaton-Dikeman No. 320 or 641; Whatman No. 3MM or 31; Schleicher and Schüll No. 589 or 602h). The paper is stretched vertically in a rack and slowly perfused from the top with buffer solution. At both sides, platinum electrodes are attached to which a direct current of 110 volts and 20–30 milliamperes is applied. Whereas in the original design of Durrum (35) the field lines traversed the square paper in the form of a half-moon, the longitudinal electrodes of Grassmann and Hannig (106) and of Strain and Sullivan (107) lead to a uniform field. The mixture to be separated is allowed to flow in from above in a fine stream; thus it moves perpendicularly to the lines of force of the electric field and its molecules are deflected during their descent to the anodic or cathodic side, depending on their charge (cf. Fig. 4). Depending on the magnitude of the deflection, the separated substance drips off the notched base of the paper into one of the twenty-eight test tubes.

Assuming that the filter paper represents only the inactive supporting medium, the path of an ion forms an angle α with the line of descent; it is

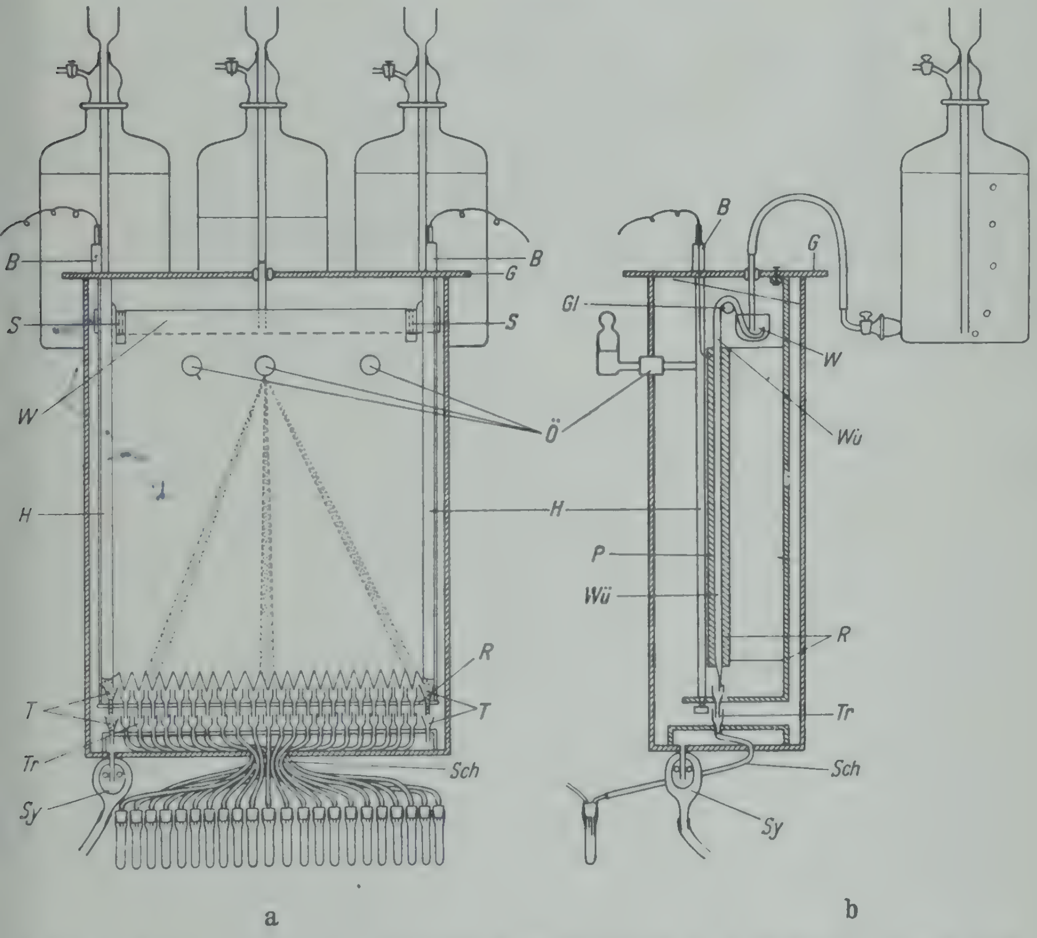


FIG. 4. Separation set-up, schematic. a. Frontal view. b. cross section, lateral view. Explanation of the symbols: R = plastic rack. W = buffer trough, S = bars, H = support arrangement, P = platinum electrodes, $Wü$ = cotton pads, Sch = rubber tubing. B = current supply boxes, Gl = glass rod, T = receiving funnels of the electrode rinsings, Tr = receiving funnels for the separated components, Sy = draining siphon, G = tightly fitting glass cover. According to Grassmann and Hannig (106).

determined by the ratio of the two components:

$$\tan \alpha = \frac{\text{migration in the electrical field}}{\text{migration caused by the fluid flow}}$$

The rack with the paper and electrodes is suspended in a Plexiglas vessel having dimensions of 200 × 26 × 7 cm., which constitutes the moist chamber.

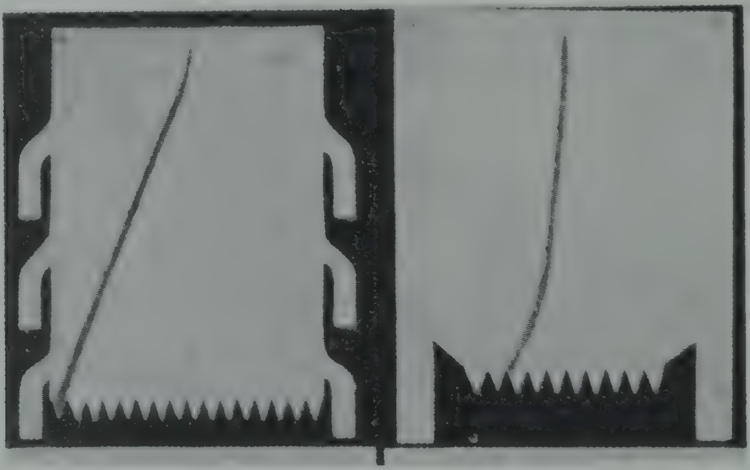


Fig. 5. Example of improved continuous paper electrophoresis. Track of bromophenol blue. According to DiCastro and San Marco (111).

The track of an ion in the paper depends on several variables; for example, the field intensity as well as the rate of flow of the buffer must be maintained exactly constant, since the ion during its electrophoretic migration is also subject to electroosmosis and rheophoresis (108). It is very difficult to circumvent the problem that the latter factors assume different values depending on the height of the paper and the distance to the electrodes. On these effects is superimposed the adsorption of the protein ions on the paper as well as their gravitation in the direction of the line of descent. Further, the zones of separation are easily disturbed by the diffusion of products of electrolysis into them; this, in turn, may lead to pH changes. If 0.1 *M* ammonium citrate is used as the electrolyte, a basic region forms along the cathode and an acid region along the anode (107), while a neutral reaction is assured only in a relatively narrow middle section. Since the separation of the proteins in 2 ml. of blood serum requires about 36 hours at a field intensity of 8 volts/cm., arrangements must also be made to keep the temperature constant. For this reason, the paper in the design of Holdsworth (109) lies on water-cooled plastic props. Similarly a water-cooling system is a feature of the apparatus of Adamik (110) in which the paper is placed around a cylinder. The new instruments aim for a uniformly rapid renewal of the buffer fluid over the entire length of the electrodes (112–117). Peeters and collaborators (118) irrigate the lateral electrodes with fresh buffer solution by means of a cascade system. Pucar (119) combines the electrophoretic separation with chromatography, while McDonald and associates (120) increase the speed of the latter method by centrifugation. The results of Grassmann and Hannig (106) indicate (cf. Fig. 6) that even continuous electrophoresis has not yet achieved a satisfactory separation of the globulin subfractions in serum.

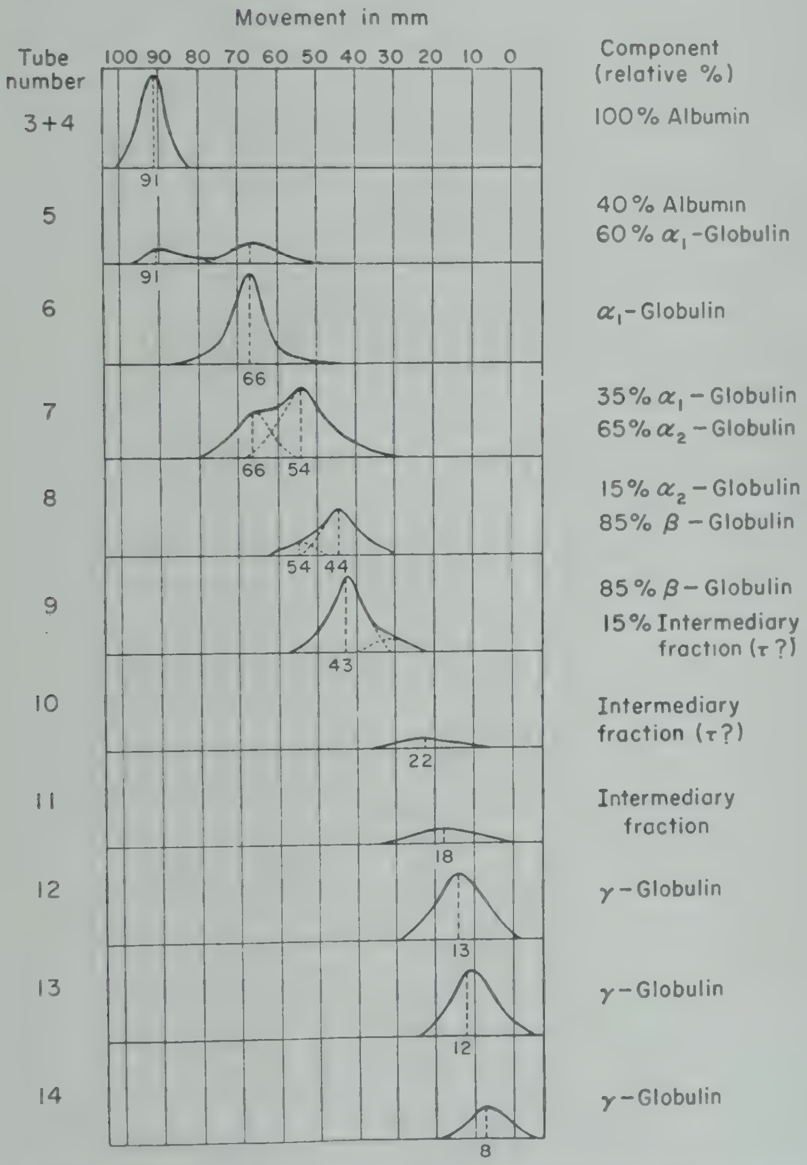


FIG. 6. Paper electrophoretic evaluation of the separated serum fractions (strip method). According to Grassmann and Hannig (106).

Further applications (121–123) have also demonstrated that a considerable expenditure of work is involved in a technique which permits only micropreparative separations to be made.

B. Brands of Paper and Pretreatment of the Paper

The paper which serves as the stationary phase of paper electrophoresis is of great importance for a good separation. Its suitability increases with a diminution in those properties which are required for absorption chromatography (124, 125). Despite the very predominantly hydrophilic structure of the cellulose molecule, a limited *physical sorption* is unavoidable with certain substances; this phenomenon may be based more on physical factors as in macroglobulin, fibrinogen, chylomicrons, and globin, or it may also have a chemical basis as in the large lipoprotein molecules. Actual selective effects have been of analytical interest in the characterization of different types of hemoglobin; in this instance, the migration velocity is influenced in part by chemosorption. That the latter often plays a part may be seen from the different electrophoretic mobility of the lipoproteins in paper and in agar gel (126). *Chemosorption* often is caused by impurities of the cellulose; these can be removed by washing in distilled water or in 1.0 M HCl. Among Whatman papers, Nos. 541, 540, 542, and 544, in decreasing order of absorptive capacity, are washed once in acid; Nos. 40, 41, 42, 43, 44, and 120 with extra-dense texture are doubly washed in acid. In the latter brands of paper the mineral content (ash) decreases to 0.01%. Merklin and Masseyeff (127) had observed that in the analysis of serum, a trace of albumin remains adsorbed on the paper. Wolvius (128) investigated this albumin tail on Whatman No. 1 paper and found an adsorption of 0.7 μg albumin per square centimeter. The determined albumin content thus is 1–2% too small; the error becomes larger as the amount of applied protein decreases. According to Eberhard (129), the paper even in these instances can saturate only a portion of all the active adsorption centers. Since cellulose contains only negatively charged groups, heteropolar linkages with basic side chains of the protein molecule are probably primarily responsible for the adsorption. In order to decrease the protein adsorption, Kraft and Biel (130) proposed pretreatment of the paper with a dilute solution of albumin.

Cellulose of good quality for analytical purposes should contain at least 96% α-cellulose, which is insoluble in concentrated NaOH (131); of the soluble portion, HCl precipitates β-cellulose, and the material which remains soluble is a small residue of γ-cellulose. The solubility of purified cellulose in water is about 13–41 parts per million. The small number of free aldehyde and carboxyl groups contained in the cellulose cannot exert any significant influence on paper electrophoresis (132, 133). However, of greater importance is its *absorptive capacity*, for the resistance which it offers the passage of the current is directly proportional to the volume of buffer fluid contained in the paper strip. In some papers, the rate of flow is somewhat greater in the direction of the rotation of the rollers, since the cellulose fibers are oriented accordingly (132). Independently of the absolute absorptive capacity of a paper, its *absorptive performance* per unit time is important for the evaluation. To test this we place 3 × 20 cm. paper strips vertically into a weighing bottle containing 20 ml. of water. After 6 minutes the paper is lifted out and the fluid loss is determined by weighing the difference. In this manner, an absorptive performance, expressed as milligrams water per minute, of 27.2 is found for Whatman 3MM, of 41.8 for Whatman 120, of 65.2 for Schleicher and Schüll 470A, of 34.6 for Munktell 20/150, and of 101.0 for 20/350 (134). Acid treatment increases the *wet strength*; this is particularly important with regard to the requirements for continuous electrophoresis. The absolute absorptive capacity (135) amounts to about 180% of the weight of the paper for rapid flow papers such as Whatman Nos. 41, 43, and 541 and to about 115% for slow papers such as Nos. 120, 542, and 544. The paper most frequently used for paper electrophoresis, *Whatman No. 1*, consists of 98–99% α-cellulose, 0.3–1.0% β-cellulose, and 0.4–0.8% pentosan; the mineral content (ash) is 0.07%. As a general rule, papers which are only thin to, at most, medium thick but endowed with dense texture yield the straighter fronts in paper electrophoresis; in contrast, thick, rapidly flowing papers with high absorptive capacity easily lead to indistinct fronts and are therefore less suitable for analytical purposes.

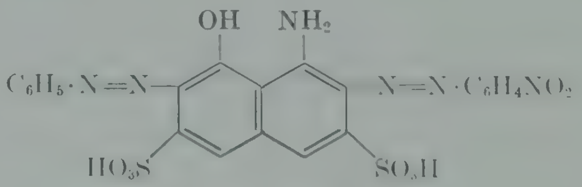
Numerous attempts have been made to alter the properties of the surface of the paper in such a way as to diminish adsorption and electroosmosis. For example, Jermyn and Thomas (136) treated the paper with periodic acid; the resulting dialdehyde groups then were coupled with hydrazine derivatives so that the surface of the paper took on a basic character. Kallee (137) treated the paper with 0.1–0.2% gelatin, serum albumin, and serum globulin and was able by this procedure to eliminate the adsorption of iodinated insulin. For paper electrophoresis of proteins migrating as cations, Monty and associates (138) treated Whatman paper 3MM first with 1% octadecyldimethylbenzyl ammonium chloride solution. In order to make the supporting medium inert, Stegmann (139) tried a polyvinyl chloride paper (Rhovyl paper of Schleicher and Schüll); electroosmosis was greatly diminished, whereas in glass filter paper it was significantly increased. Until now, procedures which change the properties of the paper by pretreatment have not found wide application. Recently, Kohn (140) successfully employed cellulose acetate, which is otherwise used for membrane filters, as the supporting medium.

C. Staining Technique

The purpose of the colorations which pathologists and histologists carry out on smear preparations or sections is to characterize qualitatively certain chemical groupings. By comparison, the aim of coloration of paper strips after paper electrophoresis is immeasurably more exacting. For example, protein stains must not only color proteins selectively but the coloration must also be proportional to protein content in a simple manner; the sensitivity must be great enough so that sections with little protein content are not lost to measurement, but at the same time sections containing a lot of protein should exhibit a color intensity which still obeys Beer's law upon direct colorimetry. Thus, much is asked of staining as an analytical procedure, the more so since all stains are only insufficiently characterized from the chemical point of view. It is easy to understand, then, that *staining and the removal of excess dye connected with it constitute the most difficult step in the technique of paper electrophoresis with regard to reproducibility.* The uncertainty is compounded when dyes are used which lack uniformity (141) or which are quite unstable in solution (142). In addition to considering these factors it is essential to carry out the staining in the right time sequence, a condition which cannot be emphasized too often in routine work.

1. Protein.

The coloration of the paper strips is independent of the choice of previous electrophoretic technique. Since protein staining has been applied and subjected to critical scrutiny most frequently, it will be discussed first. The most commonly employed dye is the diazo dye amido black 10B (= naphthalene black 12B 200) (Fig. 7), which has an elementary formula $C_{22}H_{16}O_9N_6S_2$ and a molecular weight of 572.



Amido black 10B

According to Grassmann and Hannig (33, 84, 106, 143) the dye bath consists of a saturated solution of amido black 10B in a mixture of methanol and glacial acetic acid (9:1); staining is allowed to proceed for 10 minutes with constant shaking. For staining with bromophenol blue, $C_{19}H_{10}O_5Br_4S$, molecular weight 669.6, Durrum (11, 62) gives the following instructions: 0.1 gm. bromophenol blue is dissolved with 50 gm. zinc sulfate and 50 ml. glacial acetic acid to a final volume of 1 liter; the paper strips, after drying

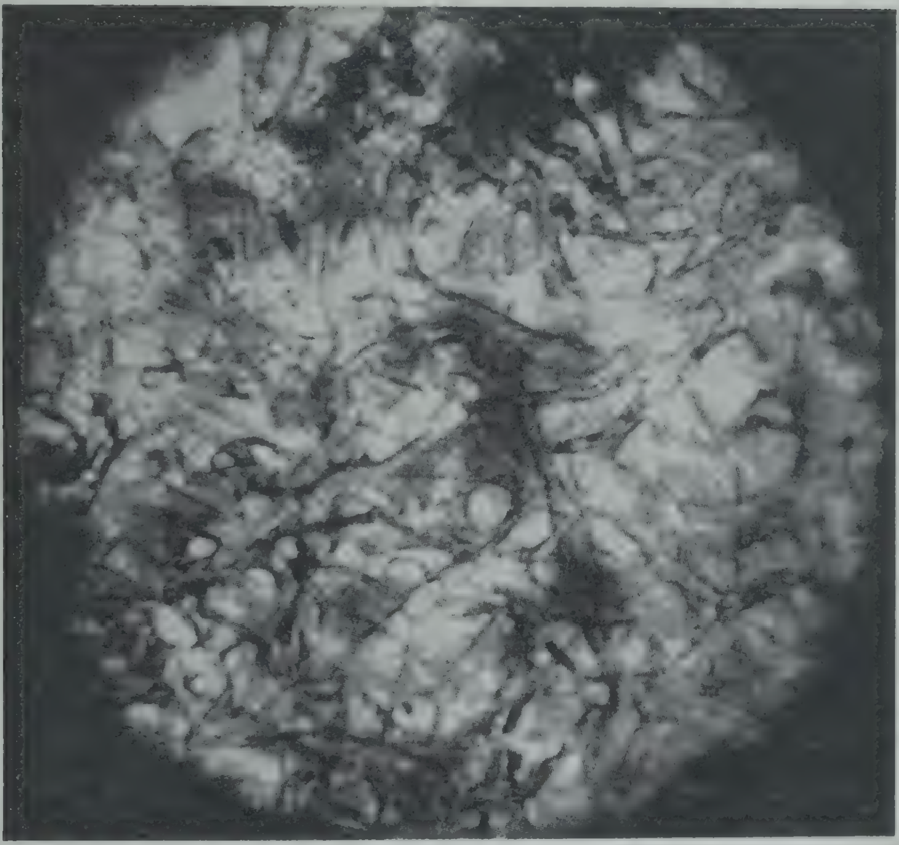


FIG. 7. Microphotograph of a protein fraction on filter paper (Whatman No. 1) stained with amido black 10B. Note the uneven distribution of the dye. According to Eberhard (158).

for 30 minutes at 110–120°C., are placed in the dye bath for 16 hours. Körver (144) and Turba and co-workers (14, 145) recommend staining with the acid wool dye azocarmine B, $C_{28}H_{20}O_9N_3S_3Cl$, molecular weight 697; staining is done for 10 minutes in a saturated solution containing 50% methyl alcohol and 10% glacial acetic acid. Other dyes which have been proposed are neococcine-acilan scarlet (146), Ponceau 2R (147), and light green S.F. (141, 148). As shown by the extinction curves (Fig. 8) (143), staining (149) of the various serum protein fractions is realized better with amido black than with the other dyes (150).

The height of the curve indicates that with amido black the absolute extinction is greater; but one must take into account the disadvantage that this dye is washed off the paper only slowly (4 hours) in baths of methanol-glacial acetic acid (9:1) even with several changes of fluid. Excess bromophenol blue is removed in three washings of 2% acetic acid during a total period of 20 minutes and then a 2-minute exposure to a bath of 2% sodium acetate and 10% acetic acid; with azocarmine, decoloration is carried out

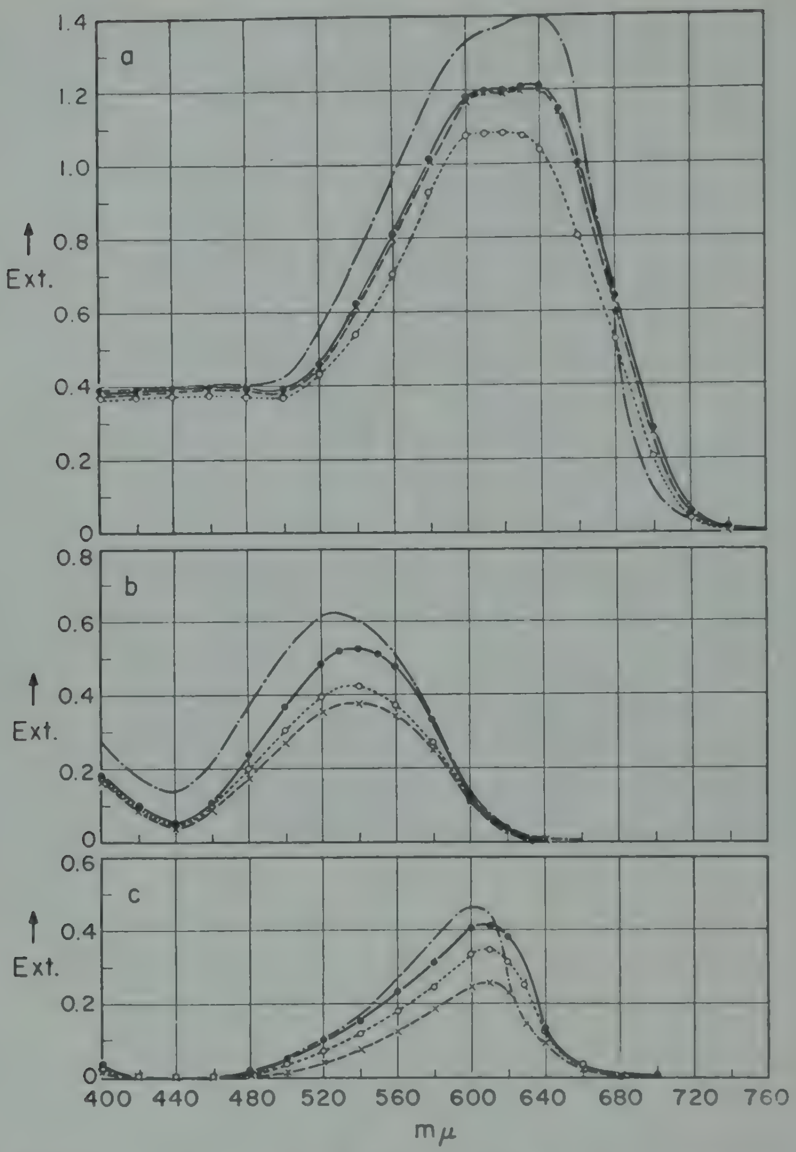


FIG. 8. Extinction curves with the dyes amido black, bromophenol blue, and azocarmine, determined on strips loaded with the same amounts of protein and stained. a. Staining with amido black 10B. b. Staining with azocarmine. c. Staining with bromophenol blue. Legends for curves: o—o—o = albumin; x—x—x = α-globulin; o··o··o = γ-globulin; —·— = curve of the pure dye on paper. According to Grassmann and Hannig (143).

in 3 washings of 15 minutes each in methanol containing 10% glacial acetic acid. The extinction values of a blank strip of Whatman No. 1 after staining with bromophenol blue, azocarmine, and amido black and decoloration are as 0.005 to 0.02 to 0.07 after a washing period of 4 hours (143). All three dyes stain serum albumin more intensely than the globulin fractions; the corresponding correction factors for amido black were calculated to be 1.4

72), 1.6 (151), 1.7 (152) to 2.2 (153). Light green S.F. (154, 155) can be vashed out of the paper completely and has been reported to stain the protein fractions uniformly (148), but its solution in acetic acid water is not stable (156); uniform staining has also been emphasized for lissamine green I.C.I.) (157).

If the evaluation is carried out by the transparency method, the differnces are smaller than in elution (cf. Section III, D, 1). Theoretical considerations of the serum protein dye-binding indicate that adsorption is nvolved (130, 158). It cannot be assumed that this adsorption is equally strong for all protein fractions. Consequently, the elution effect also differs n magnitude.

2. *Lipids and Lipoproteins*

Since the lipids of blood serum and other body fluids are very heterogeneous in composition, their staining represents the result of complex nterrelationships; indeed, the solubility of the various components is influenced by their relative quantitative proportions, so that one should not xpect a quantitative answer. This reservation is even more important for sera whose composition is greatly altered in various diseases. Nonetheless, he scope of paper electrophoresis was enlarged significantly when Swahn (159) demonstrated in 1952 that lipids and lipoproteins could also be stained on paper strips. For this purpose, he poured 100 ml. of 60% ethanol ver 0.1 gm. Sudan black B (National Aniline) and slowly heated to boilng; heating was stopped immediately on reaching the boiling point; after ooling, the mixture was filtered twice through hard filter paper. The paper strips were placed into the filtrate for 3 hours and then were freed of excess stain by three washings of 15 minutes each in 100 ml. of 50% ethanol. Oil lue N (National Aniline) (62), Sudan III (160), lipid red 7B (Ciba), and Sudan IV (161) may be used in a similar manner (Fig. 9). Another freuently used dye is oil red 0 (35, 162), whose saturated solution in boiling bsolute ethanol is filtered after cooling and brought to 60% ethanol with vater. The paper strips are left in this solution overnight and then washed vith running water. For direct colorimetry, the strips after drying are athed in glycerin and made transparent. For elution, the appropriate secions of the paper are cut out and placed for 1 hour with intermittent agitaion in 6 ml. ethanol containing 20% glacial acetic acid; colorimetry on the luate is carried out at wavelength 540 mμ (155). Lipid staining with osmic cid (163) or rhodamine G (164) has been carried out in isolated instances. For all these dye baths the rule holds that it is best to use them as freshly repared as possible; also, the paper strips must be kept from sliding over ach other in the dye bath.

In order to characterize the *cholesterol content* of the sections of paper

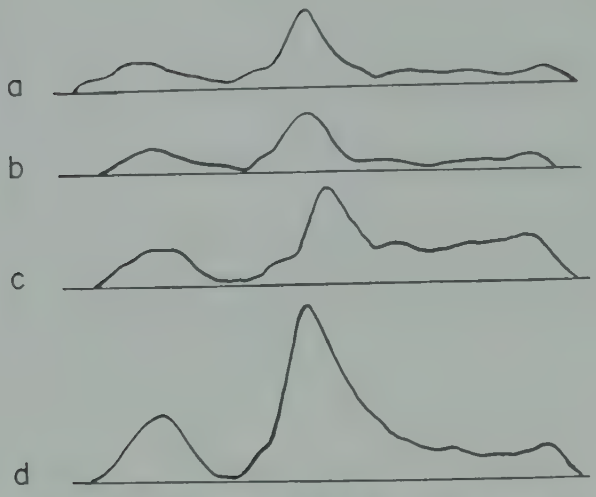


FIG. 9. Comparative representation of the separation of lipoproteins of the same serum obtained with different staining techniques. *a*, Sudan II, oil red O; *b*, Sudan III; *c*, Sudan IV; *d*, Sudan black B. According to Gronow (231).

as well (165), a paper strip is run in parallel with the one to be stained for lipid; the latter indicates the sections of the paper according to which the uncolored strip is to be cut. The finely comminuted papers are placed for 10 minutes in 5 ml. of a mixture of methanol and chloroform (1:3). The fluid is poured into test tubes and fat extraction is then repeated twice with another 3-ml. portion of the mixture. The combined extracts are evaporated at 90°C. and the dry residue is shaken vigorously with 3 ml. of acetic acid. Then 2 ml. of the color reagent is added; this is a solution of 1 gm. ferric chloride, $6H_2O$, in 10 ml. of glacial acetic acid, 1 ml. of which is mixed with 100 ml. concentrated H_2SO_4 immediately before use (166). Colorimetry is then carried out with a green color filter against blank values obtained with sections of paper of corresponding size.

3. Gluco- and Mucopolysaccharides

In staining these groups of substances, Köiv and Grönwall (167, 168) followed the procedures used by Hotchkiss (169) in pathology. The difficulties inherent in this staining method are related less to the differences among the various carbohydrate components than to the insufficient stability of the coloration on the paper strip. The blank coloration of the paper, in particular, is so variable that the divergent blank values often make good reproducibility impossible. In many instances, the coloration can be valuable on a comparative basis, but without any attempt at quantitative assay. To obtain reliable weight determinations, microchemical techniques (170) are essential (171-173); with their aid, the carbohydrates can be estimated in the eluates of preparative electrophoresis (174).

A stock solution of periodic acid Schiff (PAS) reagent is prepared by dissolving 0.5 gm. basic fuchsin in 100 ml. of boiling water and then adding 100 ml. 1 N HCl and 0.5 gm. potassium metabisulfite. During storage overnight, the color changes from red to light brown. At this point, a spoonful of activated charcoal is added and the mixture is filtered; the filtrate should be colorless. For staining (175) the stock solution is diluted 1:20 with water; the reddish-violet coloration should appear within 5 minutes. The following must also be prepared:

(1) *Solution A* (can be kept for 1 week): periodic acid, 400 mg., 96% alcohol, 95 ml., 0.2 M Na acetate, 5 ml.

(2) *Solution B* (can be kept for 1 month): potassium iodide, 1 gm., Na thiosulfate 1 gm., distilled water, 15 ml., 96% alcohol, 35 ml., 2 N HCl, 0.5 ml.

Procedure:

(1) The dry paper strips are bathed for 5 minutes in solution A, then washed in running water, and finally washed thoroughly in distilled water.

(2) The paper strips are bathed in solution B until the yellowish coloration disappears; washing as above.

(3) The strips are placed in the diluted stock solution until the red-violet bands at the location of α- and β-globulin are clearly developed. Wash first in distilled water, then four times in baths of distilled water through which SO_2 has bubbled for 30 seconds and which was acidified with a few drops of 2 N HCl.

The completely stained papers are blotted with blotting paper and dried at room temperature. A slight lilac coloration of the background should disappear during drying. If the last washes are too acid, the stain suffers upon storage. With normal serum proteins, more intensely colored bands appear at the locations of α_1-, α_2-, and β-globulin, while albumin and γ-globulin stain only slightly; the most intense color is shown by α_2-mucoprotein. For the exact localization of the colored bands, the parallel strip with protein staining can be used (176–178). If the lipid staining is also carried out, this "triad staining" characterizes the three important groups of compounds (175, 179). (See Fig. 10.)

4. Check of the Coloration

Since the measurements depend on the coloration which has been achieved, the question arose repeatedly whether the *intensity of coloration* is proportional to the *concentration of the substrate*. Pertinent model experiments were deficient *a priori* in that they were made with relatively simple model substances (180, 181), whereas the biological substrates pos-

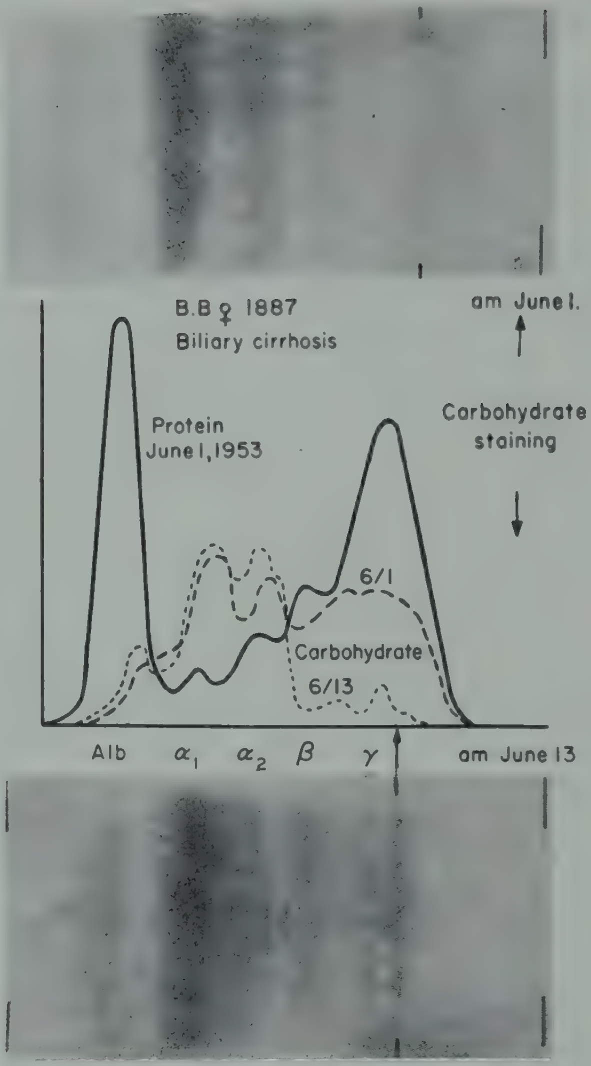


FIG. 10. Example of PAS-staining of a separated serum. According to Wunderly and Piller (179).

sess an extremely heterogeneous structure. The obtained correlation, indeed, was usually unsatisfactory. However, in order to achieve a control to which the result of staining and decoloration by means of Sudan black or oil red can be related, Wunderly and Wieme (182) developed the *triglyceride standard*. This is based on the fact that triolein, lecithin, and corn oil are stained by Sudan black as intensely as the lipoproteins of serum. A solution is prepared of 300 mg. % triolein (Merck) and of 100 mg. % tristearate (British Drug House) in chloroform; 0.01 ml. of this solution is applied to the dry paper strip (after paper electrophoresis) in the forms of dots on both sides of the paper strip. As a staining control of the total lipids, 0.01 ml. of 1:1 diluted serum can also be applied next to it. The coloration with Sudan black (183) is eluted with ethanol plus 20% glacial acetic acid and determined colorimetrically. If a series of normal sera are assayed in this manner, an average reference value is obtained. The color value of the normal lipoproteins of serum is thus standardized; in this way, technical deficiencies such as inaccurate elution or atypical lipid colorations as a consequence of disturbances in lipid metabolism are easily recognized and evaluated accordingly.

In the same vein, we have proposed the synthetic polyethyleneimine, $-[CH_2\text{-}CH_2\text{-}NH]_n-$, as a reference substance for protein staining (184). This is a linear colloid of approximate molecular weight of 30,000–40,000, which has been made available commercially by the Badische Anilin- und Sodafabrik in Leverkusen (Germany) under the name "Polymin P." The marketed sol is diluted to 50 mg. % with distilled water and 0.02-ml. quantities are dropped onto the paper after paper electrophoresis to the left and right of the protein bands. The dried strip can then be stained with amido black, azocarmine, bromophenol blue, or light green. Amido black exhibits a much greater color index with this material than with serum protein fractions; this staining behavior may be explained by the large number of charges on the colloid molecule (Fig. 11).

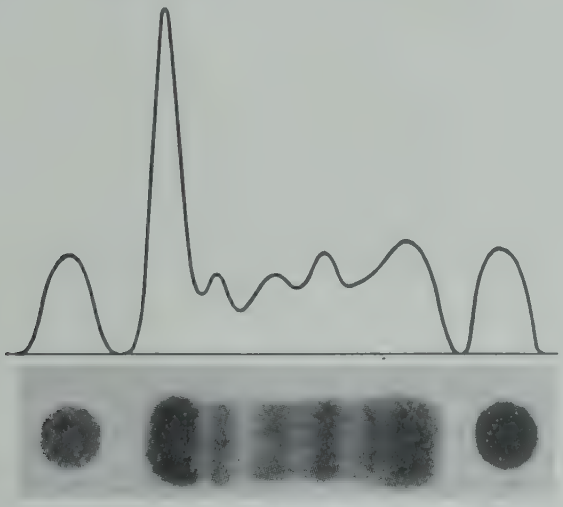


FIG. 11. Check on amido black staining with Polymin standard. Assay with the Zeiss extinction recorder II. According to Wunderly (185).

Each application of 10 μg. Polymin P gives on staining with amido black a spot of 3.27 cm.^{2} average size, according to estimations of the bell-shaped curve (Zeiss extinction recorder II), therefore, an average of 3.058 μg. Polymin P occupies 1 cm.^{2} or 1 μg. occupies 0.327 cm.^{2} on Whatman No. 1 paper. Comparison with these standard values permits the evaluation of the technically unavoidable variations of staining with amido black or other dyes on Whatman No. 1 (185). On the basis of the *Polymin standard* it was possible to determine the area occupied by 1 μg. protein on 10 different papers, as well as color intensity and color index (186); these measurements yielded new material constants for these papers.

D. Methods of Estimation

1. Methods and Instruments

A reliable estimation presupposes an unequivocal separation with clear-cut straight fronts. In addition to quality of paper (187) and current relationships, the choice of the ionic strength of the buffer system plays an important role in the separation (48, 49, 52). The estimation of stained paper strips can be carried out by the *elution method*, the *reflection method*, or by *direct photometry* (transparency method). Elution can be carried out with equipment available in any laboratory and is employed to advantage if paper electrophoresis is carried out only occasionally, e.g. for purposes of control; in contrast, direct photometry is particularly useful for routine determinations.

a. Elution method. The stained strip is cut transversely into small pieces 5 mm. wide; one unstained little strip from each end of the paper provides the blank value. This blank must be redetermined each time since it varies with the dye vat and washing procedure. Then, the appropriate solvent (cf. Section III, C, 1, 2, and 3) is used for elution, for example, 1 N NaOH in 50% ethanol plus 0.25% Versene [ethylenediaminetetraacetic acid, which complexes traces of metal ions (188)] for the proteins of serum. After exhaustive elution, the optical density is measured in a colorimeter with photocell and light filters at the wavelength which is appropriate for the dye. Beer's law (189) which is the basis of the measurement states the dependence between the light intensity I_0 of the blank paper value and the dye-dependent decrease of the intensity I as an expression of the extinction

$$E = \log \frac{I}{I_0} = -\epsilon c d$$

where ϵ is the specific extinction coefficient, c the concentration of the absorbing material in grams, and d the thickness of the cuvette in centimeters; this is a limit law for high dilutions, since it would be strictly valid only if ϵ were independent of concentration. At greater concentrations of the dye, therefore, the increase in E is also more pronounced since van der Waal's forces come into play.

Optical density, then, is

$$D = \epsilon c d = \log 100 - \log T$$

where T is the percentual transmission (190). To elute amido black quantitatively from the paper strip, Oosterhuis and associates (191, 192) extract it at 65°C. in a Soxhlet extractor with water containing 1% glacial acetic acid. If it is desired to represent the results of the elution as a curve, the strip must be cut into a large number of sections; each of these yields one point on the curve. For clinical-chemical assays it often suffices to carry out the separation and elution in fractions.

b. Reflection method. The application of direct reflection photometry for the evaluation of colored paper strips has been described in detail by Owen (193). Earlier studies (194, 195) had shown that a measurement can be based on the decreased intensity of the light reflected from the colored zones. Further applications, however, have remained infrequent (196, 197).

c. Direct photometry. For the transparency method, the stained and dried paper strips must first be made transparent. For this purpose, glycerin has been proposed (198); it lowers the blank absorption of the paper to one-tenth and is employed in the assay of lipoproteins. Other suitable reagents are a mixture of paraffin oil and α-bromonaphthalene with a refractive index $N_D = 1.51$, which corresponds approximately to the index of refraction of cotton fibers; benzyl alcohol with $n_D = 1.539$ (199); methylsalicylate with $n_D = 1.535$–1.538 (200); Caedax (Bayer) (201); and Herbopanlack (Herbig-Haarhaus). The latter raises the light transmission of Whatman No. 1 and of Schleicher and Schüll 2045a from 45% to almost 100% compared to oil transparency of 80% (202); it transforms the paper strip into a transparent dry film (203). Anisol with $n_D = 1.518$ (204) also has the advantage that it evaporates easily. In the dye-dependent decrease of light, only absorption is to be measured and not scattered light; in the dry film there is greater assurance of a linear relationship between color density and extinction than in the paraffin-containing fluids. By this procedure one also avoids residual air bubbles which introduce a considerable error into the measurements. Attempts to avoid staining and to measure the ultraviolet absorption of the proteins directly (205) have encountered difficulties caused by the scattered light and the large absorption of cellulose. Similarly, graphic and mathematical methods for the evaluation of strips (206–208) have failed to find wide acceptance because of their time-consuming nature or mathematical requirements.

At present, *extinction measurements* are carried out predominantly either with instruments which measure only the extinctions, which then are plotted on millimeter-paper, or with automatic recording devices. Routine work requires time-saving devices and elimination of subjective sources of error by automatization. It was shown in Section III, D, 1 that according to the Lambert-Beer law extinction of a colored solution is given by the product of a constant, the color concentration in grams per liter, and the thickness of the layer. Applied to a transparent strip (143), the concentration c is

$$c = \frac{a}{r}$$

where v represents the total volume of the solution and a the weight of material dissolved in it. If v is replaced by the product of the area of the strip f perpendicular to the transmitted light beam, and the thickness of the layer d, one obtains the relationship

$$E = k\frac{a}{fd}d = k\frac{a}{f} = kcd$$

The first usable instrument was based on the preliminary studies of Grassmann and Hannig (33) and Grassmann *et al.* (84) (see Fig. 12). The transparent paper, free of bubbles, is placed between two glass plates; the latter are introduced into a slide which is moved in 1-mm. steps over a slit of 1-mm. width (Bender and Hobein, Munich 15, Germany).

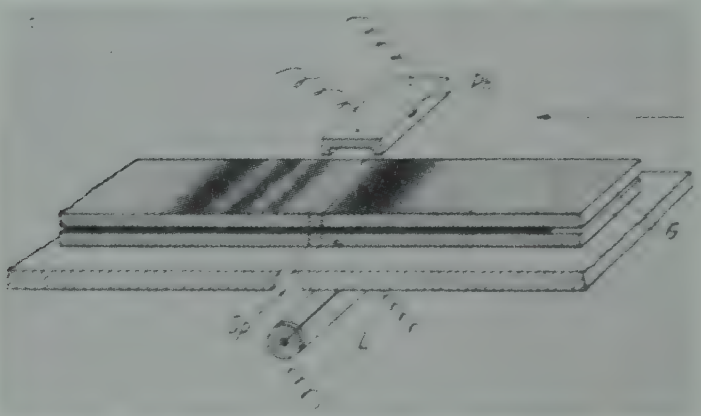


FIG. 12. Arrangement for photometric determination according to Grassmann and associates (33, 84). G = glass plates, Sp = slit, Ph = photocell, L = filament lamp.

The slit diaphragm (Sp) is illuminated by the 6-volt, 10-watt filament lamp L (201, 209, 210). By this arrangement scattered light is prevented from reaching the photocell (Ph), and the light beam, decreased by absorption in the particular color intensity, is allowed to register. The light element is connected to a galvanometer whose deflections provide a measure of the magnitude of absorption. The measurement is improved if a light filter G5 (Schott and Gen., Germany) is placed in the light path; it filters out the wavelengths below 550 mμ and passes the maximally effective light in the range of 590 to 630 mμ; if the light is not monochromatic, there are deviations from the Lambert-Beer law so that the relation between extinction and amount of dye is no longer linear. If monochromatic measurements can be carried out, the maximum for amido black 10B and bromophenol blue is at 578 mμ, that for azocarmine and ninhydrin at 546 mμ (211). This sort of measurement is made possible by the auxiliary instrument for the photometer "Eppendorf" (Netheler and Hinz, Hamburg-Eppendorf, Germany), which employs a mercury spectral lamp as the light source; measurements are made with a photocell and a galvanometer with tube intensifier. These advantages are also realized in the Photovolt Densitometer (Photovolt Corp., 95 Madison Avenue, New York, 16). Some of the most widely used manual analyzers have now been mentioned. For the planimetric evaluation of the resulting curve, a planimeter with a movable lens and an integrating counting mechanism is well suited. The curve of, say, a serum protein consists of several Gaussian distribution curves. If these overlap to some extent (206) (e.g. albumin and α_1-globulin), a straight line is drawn which is parallel to the axis of the abscissa and lies at the halfway mark in height; the portions of the curve lying above this straight line can then be calculated according to

$$J = kca$$

where J is the area, k is a constant ($k = \sqrt{\pi}/2\sqrt{\ln 2} = 1.064$), c is the height, and a is the width of the Gaussian curve at the halfway point in height. The determination of a and c on the curve, therefore, is sufficient for the calculation of the area J. The area can be determined also by weighing the single sections of paper of excised Gaussian curves.

The automatic analyzer according to Eberle-Edelmann (Bender and Hobein, Munich 15, Germany) (212) employs a mercury spectral lamp with corresponding filters; the single extinctions are measured automatically millimeter by millimeter and fixed on a paper strip by punching; this sequence of punches describes the course of the concentration curve. A compensatory measurement principle permits the simultaneous summation of the single extinctions by a counting mechanism. The automatic "extinction recorder" (C. Zeiss, Oberkochen, Germany) also shifts the measurement to the extinction maximum by a suitable combination of filters. This instrument, in which the compensation principle has also been realized, writes a continuous extinction curve by means of an ink pen. The automatic integration of the areas circumscribed by these curves is carried out by the "Elphor-Integraph" (Bender and Hobein, Munich 15, Germany) (213) and by the "Analytrol", Spinco Model R (Spinco Division, Beckman Instruments Inc., Belmont, California). Both instruments simultaneously draw the extinction curve and yield the objective values for the area of the separate serum protein fractions in numbers (214, 215).

To obtain a comparison of the scatter, Grassmann and Hannig (143) assayed five different strips of a separated normal serum in four instruments (Table IV).

This comparison indicated a certain superiority of the automatic analyzers over the visual readings. Other photoelectric analyzers have been developed by Crook and collaborators (216), Knapp and Sieler (217),

TABLE IV

Comparison of Results of an Analysis in Quintuplicate on a Normal Serum[a]

	Relative percentages and error margin as determined with:				
Fraction	(a) Elphor-H	(b) Eppendorf colorimeter	(c) Edelmann instrument	(d) Zeiss extinction recorder	Average planimetry error
Albumin	62.8 ± 1.6	65.1 ± 1.0	63.4 ± 0.5	63.0 ± 0.8	±0.3
α_1-Globulin	5.8 ± 0.6	5.5 ± 0.6	5.7 ± 0.3	5.8 ± 0.2	±0.2
α_2-Globulin	6.5 ± 0.5	6.1 ± 0.4	6.6 ± 0.4	6.8 ± 0.4	±0.2
β-Globulin	8.3 ± 0.5	7.8 ± 0.6	8.4 ± 0.3	8.2 ± 0.6	±0.2
γ-Globulin	16.6 ± 1.1	15.6 ± 1.1	15.9 ± 0.5	16.2 ± 0.7	±0.2

[a] According to Grassmann and Hannig (143).

Weicker (210), Rebeyrotte (218), Schulz-Methke (219), and Mackay and associates (220). Instruments with electronic conversion of the absorption measurement into an extinction measurement have been described by Laurence (221) and Poulik and Pinteric (222).

2. Reproducibility and Size of Error

It is easy to understand, in view of the extensive application of paper electrophoresis and of the significance of its results for diagnosis and prognosis, that many attempts have been made to establish the variation of the method (150, 223–225). We have shown (226) that this purely methodological range of error approaches the biological variation; the factors which cause it may now be listed. Since human blood serum has been the most widely studied substrate, it was also employed for the methodical examination of the reproducibility. From the analytical point of view, a standardized preparation of crystallized serum albumin would be preferable to the heterogeneous body fluid; due to the relatively small proportion of globulin subfractions in normal sera, 60–65% being serum albumin, a significant *difference in the color density* arises (Fig. 13). Since, moreover, albumin is more intensely stained, it is difficult to achieve the desirable linear dependence of concentration and extinction. If the dye-binding capacity of serum albumin is indicated by 1, the correction factors for γ-globulin, according to Pezold and Peiser (151), are 1.1 for bromophenol blue, 1.6 for amido black, and 1.8 for azocarmine; since the characteristic of the optical system employed differs from investigator to investigator, it is hard to compare even analogously carried out determinations (226–228); on the other hand, a majority of investigators find that the elution technique more effectively overcomes this difficulty. But since it involves more work, the direct pro-

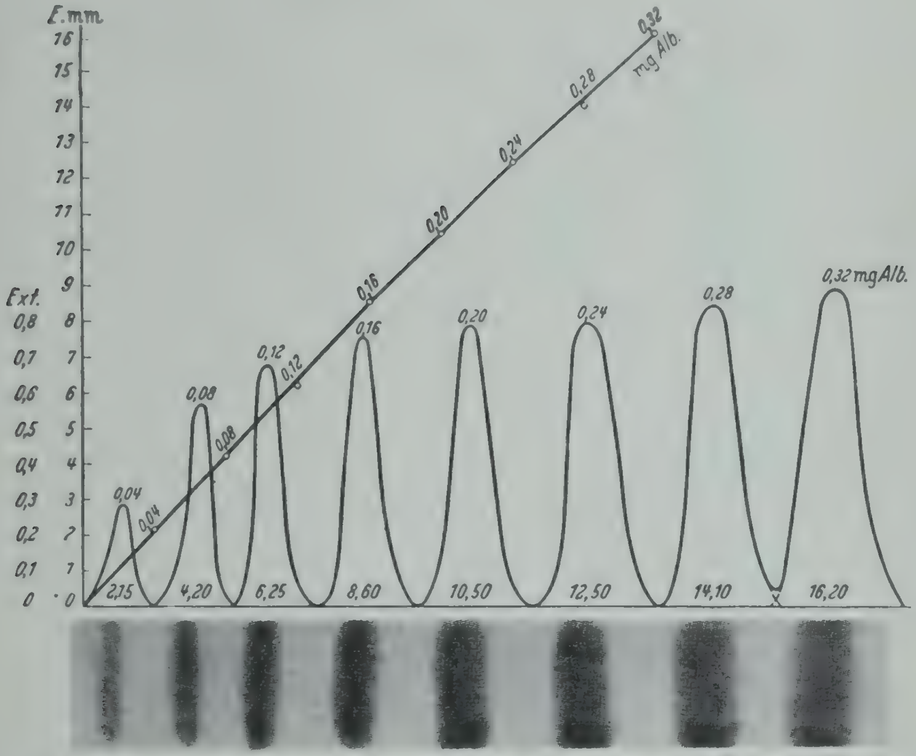


FIG. 13. Calibration curves with serum albumin, according to Grassmann and Hannig (143). Bottom: strip loaded with increasing amounts of albumin and stained with amido black 10B. Above, its photometric evaluation. The numbers within the Gaussian curves are the integrals of the area obtained by planimetry. o—o—o is the calibration curve determined from the values of the area.

cedures are preferred in the routine laboratory. From the clinical side it is often stressed that only fairly sizable alterations in protein composition can be considered really significant in any single case; the modern direct methods fully satisfy these requirements for precision. According to Heide and Biel (229) the elution effect can be improved by the addition of 1% Tween 80 to the methanol containing 10% glacial acetic acid. It is also possible to lower the limit of error by the addition of 0.005% albumin (commercial preparation, Behringswerke, Marburg, Germany) (230); this causes a preimpregnation of the cellulose with protein and thus decreases adsorption. In this case, the end point of the photometric measurement at the end of the γ-globulin fraction coincides with the null point of the unused strip. This residual extinction should not exceed $E = 0.04$, and it provides at the same time a measure of the adsorption which has taken place. It is obvious that the latter depends greatly on the nature of the proteins to be separated; such predominantly linear proteins as fibrinogen, globin, and tissue protein are much more strongly adsorbed than the hydrophilic, corpuscular serum proteins.

To determine the contribution of the separate operations to the total error (direct photometry, Elphor H analyzer), Grassmann and Hannig (33) subjected the same serum ten times to paper electrophoresis and evaluation (cf. Table V, columns 1–3); further, a stained strip was measured photometrically and evaluated ten times; finally, the results of a single photometric determination were transferred ten times to millimeter-paper and subjected to planimetry after dissection into Gaussian curves.

TABLE V

STANDARD DEVIATIONS[a,b]

Fraction	1	2	3
Albumin	±1.2	±0.4	±0.3
α_1-Globulin	±0.6	±0.3	±0.2
α_2-Globulin	±0.7	±0.3	±0.2
β-Globulin	±0.6	±0.3	±0.2
γ-Globulin	±1.0	±0.4	±0.2

[a] From Grassmann and Hannig (33).

[b] See text for details.

Column 1 gives the total error of the method; the difference between columns 1 and 2 (Table V) corresponds to the part of the error caused by the actual electrophoretic separation, the staining of the paper, and the inhomogeneity of the paper. This part of the total error, then, amounts to 50–65%, and for this reason, in Fig. 14, we give an example of a good separation which was even carried out on 8 different days.

The difference between columns 2 and 3 (Table V), on the other hand, indicates the size of the error caused by recording and evaluation, and is much smaller. The composition of the total error thus established is confirmed clearly by the comparison (Fig. 15) of three evaluations of a separation in which the fronts traveled in the form of a half-moon.

Finally, a comparison may be presented (Fig. 16) which was made by Sundermann and Sundermann (233) by evaluating the results of normal sera in the Elphor instrument and automatically in the "Analytrol." The differences observed increased in the sequence β-globulin < α_1-globulin < α_2-globulin < γ-globulin < albumin; the automatic instrument determines less albumin and correspondingly more of the globulin subfractions. All these comparisons demonstrate the desirability of a better standardization of the procedures. The inquiries of Peeters and Vuylstecke (234) and of Caroli and Charbonnier (235) represent a start in this direction

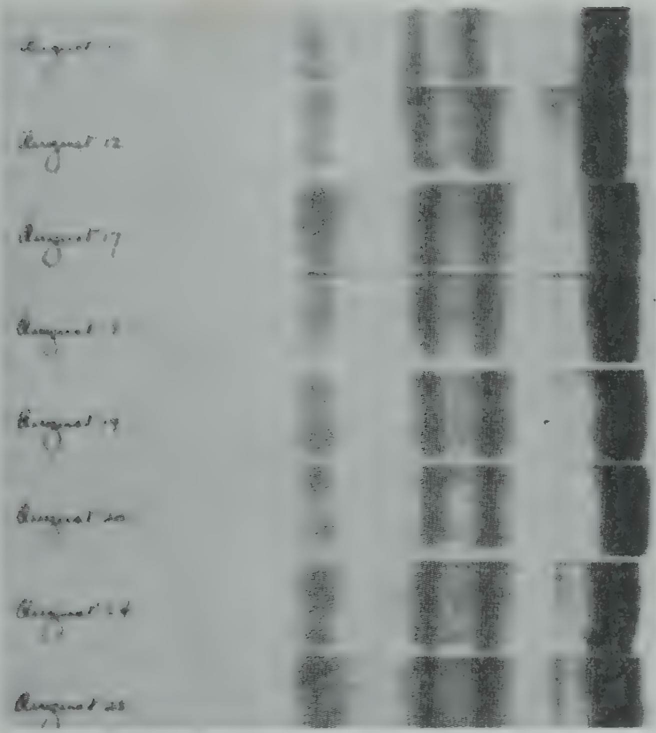

August 12
August 17
August 19
August 20
August 24

FIG. 14. An example of the reproducibility of the separation and staining of a serum. According to Williams *et al.* (83).

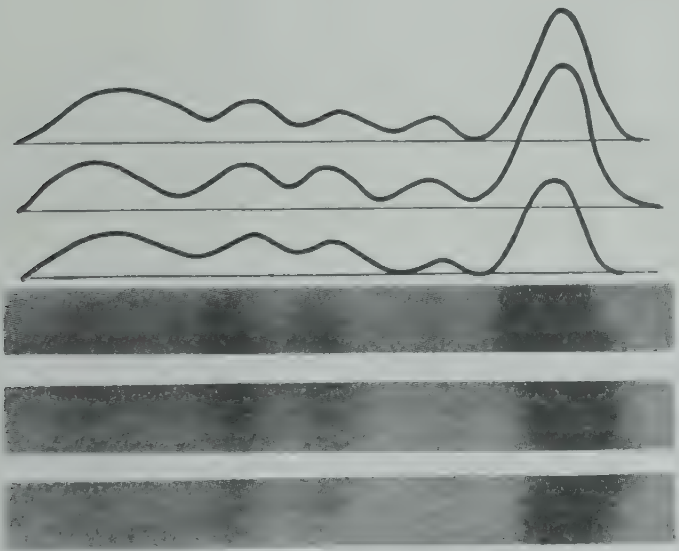


FIG. 15. Influence of insufficiently straight fronts on the evaluation: bromophenol blue staining. According to Azerad *et al.* (232).

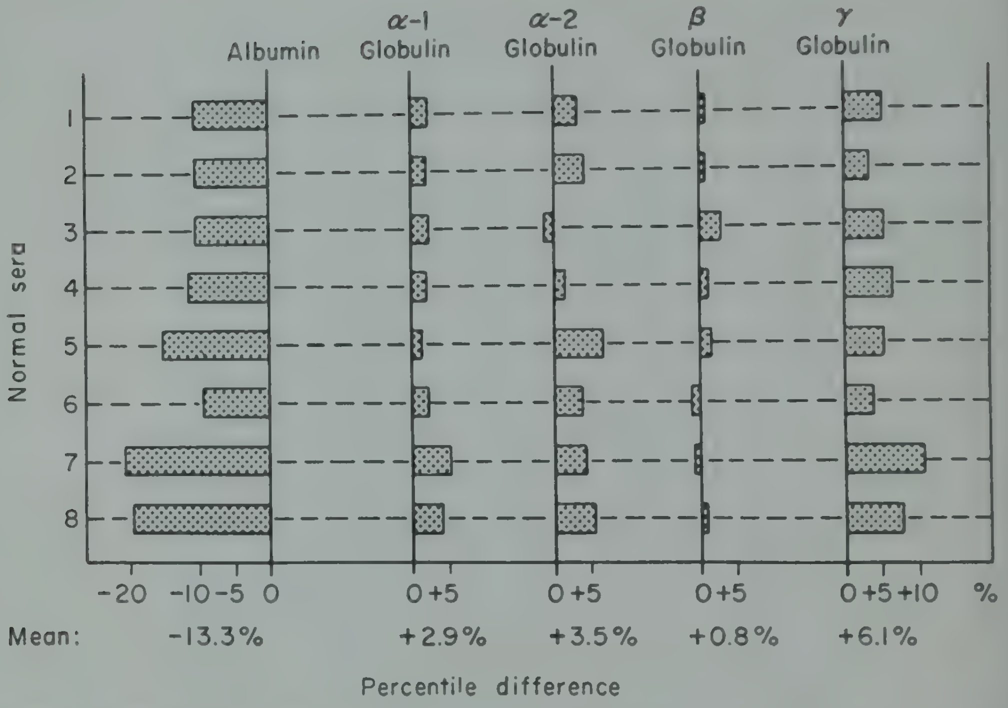


FIG. 16. Percentile differences after the assay of electrophoretically separated normal sera in the Elphor apparatus and in the fully automatic "Analytrol" instrument. According to Sundermann and Sundermann (233).

3. *Comparison of the Results with Free Electrophoresis (Tiselius)*

Since the method of determination of free electrophoresis in the design of Tiselius-Philpott-Svensson is fundamentally different from that of paper electrophoresis, only a partial comparison of results is possible. It is known, for example, that in the measurement of refraction (free electrophoresis) the lipid component of β-globulin can cause an "anomaly." Therefore, Trautman (236) proposed that free electrophoresis be carried out in two ways, i.e. on serum and on defatted serum; the lipids were to be characterized by flotation in the ultracentrifuge. Ott and collaborators (237), in a comparative test of both methods found the principal difference, 5.2%, in β-globulin, as compared to 1.1% in α_1-globulin and 2.2% in α_2-globulin; it may be assumed that these differences would be even greater in pathological sera. Table VI shows the standard deviation of the error range as obtained in free electrophoresis, but with different optical systems.

A comparison of the results with Table V shows that the reproducibility of paper electrophoresis is largely analogous, although a difference of practical significance resides in the fact that paper electrophoresis as a routine method is carried out by technicians whereas free electrophoresis is generally employed as a scientific method (single determination) by inves-

TABLE VI

STANDARD DEVIATION OF ERROR RANGE IN FREE ELECTROPHORESIS

Reference	Procedure	Standard deviation in %					
			Globulin				
		Albumin	α_1	α_2	β		γ
Dole (31)	Tiselius	±2.8	±0.7	±1.3	±1.9	±0.6	±2.5
Armstrong (238)	Tiselius	±1.3	±0.5	±0.8	±1.6	±0.6	±0.7
Everbeck (239)	Antweiler	±2.49	±1.75[a]		±0.29		±1.92

[a] Total α-globulin fraction.

gators of academic rank. That the automatization of paper electrophoresis is a technical success may be seen in Table VII.

TABLE VII

COMPARISON OF MOVING BOUNDARY RESULTS WITH PAPER ELECTROPHORESIS WITH QUANTITATION BY "ANALYTROL" SCANNER AND "ELPHOR" PHOTOMETER[a]

Method	Albumin	Globulins			
		α_1	α_2	β	γ
Means of 5 normal sera[b]					
Free electrophoretic analyses	55.7	5.1	8.3	15.9	15.0
"Analytrol" scanner	55.7	3.1	9.1	15.9	15.1
"Elphor" Photometer	56.0	4.0	9.1	14.0	16.9
Means of 10 normal sera[c]					
Moving boundary (Tiselius)	57.7 ± 2.7	4.5 ± 1.1	7.2 ± 1.1	15.3 ± 2.4	15.3 ± 1.5
Elution (bromophenol blue)	58.1 ± 4.7	3.4 ± 1.0	8.8 ± 2.0	11.9 ± 2.2	17.8 ± 1.9
Scanning (Amido black 10b)	49.1 ± 3.3	6.0 ± 1.9	10.5 ± 1.5	13.3 ± 1.8	21.1 ± 1.9
Scanning (bromophenol blue)	43.8 ± 4.0	5.6 ± 0.8	12.2 ± 2.1	15.6 ± 1.4	22.7 ± 3.0
"Corrected" scan bromophenol blue)	52.4 ± 5.3	4.1 ± 0.5	10.2 ± 2.3	13.8 ± 1.5	19.7 ± 2.8
"Analytrol" (bromophenol blue)	58.6 ± 7.0	3.4 ± 0.7	8.0 ± 2.7	12.5 ± 3.2	17.6 ± 3.1

[a] Means and standard deviations expressed as per cent of total protein.

[b] Mackay (214).

[c] Comparison of moving boundary and filter paper electrophoretic analyses of ten normal sera (Klatskin *et al.*, 215).

As the comparison demonstrates, the results of free electrophoresis agree to a large extent with those of paper electrophoresis (particularly with the "Analytrol" procedure). In the pathological samples of a clinic the deviations of the two methods may rise to ±4.5% (33, 217, 220). If the better comparable normal serum values in Table VII are compared, the largest differences in content are seen in α_1-globulin because it is frequently separated incompletely in all electrophoretic methods; the results with elution and with the "Analytrol" instrument, on the other hand, are in remarkably close agreement. Since automatization also eliminates some subjective sources of error, the method is assured of the desired distribution. As its use spreads, the determinations of the various clinics and institutes also grow more comparable.

IV. CONCLUSION

Paper electrophoresis, if carried out carefully and particularly with automatic technique, has gained a reliability of reproduction of the results which makes it the peer of the other electrophoretic methods. This statement refers primarily to the separation and quantitative determination of protein mixtures. Lipoproteins and mucoproteins can be localized and characterized appropriately, but for quantitative determinations microchemical methods continue to hold the fore.

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Chapter 6

Zone Electrophoresis in Various Types of Supporting Media

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I. Introduction

One of the most active fields in electrophoresis in recent years has been that concerned with the development of new supporting media for electrophoretic separation. Most of this work began through an effort to avoid some of the limitations of filter paper electrophoresis and to obtain larger-scale separation. Preparative electrophoresis on a moderate scale has become possible through the use of such materials as glass beads, granular starch, powdered cellulose, and polyvinyl particles. Numerous methods of utilizing the packed media have also been developed. Various trough systems and packed columns have found wide application, while rectangular chambers utilizing the principle of continuous flow electrophoresis show considerable promise as preparative tools. In addition some of the new media have proved to have selective advantages as analytical procedures. This has been particularly true of the gels, and although these were really the first media used in electrophoresis (1, 2) their potential was not realized until very recently. The separatory power of some of the gels exceeds that obtained with other supporting media or in free solution. This appears to be due in large part to the addition of a new major parameter to the electrophoretic separation, that is, separation on the basis of size and shape, in addition to electrical charge.

II. Methods

A. Open-Block or Trough System

This is the commonest procedure for using various granular supporting media and consists simply of packed material on a glass or Lucite plate which in turn rests on the lips of electrode vessels (3, 4, 5). The method as used in the authors' laboratory will be described in some detail (see Fig. 1). In some cases the medium is self-supporting as with granular starch, but in most instances it is necessary to have supports at the sides. These (Lucite bars) can be placed directly on the glass plate in the desired position and wet polyethylene sheeting fitted over them as well as over the bottom of the glass plate, thus forming a trough which is readily disassembled at the end of the experiment. Supports at the ends have not been found necessary. The supporting medium with buffer is then poured into this troughlike arrangement or into some other especially constructed

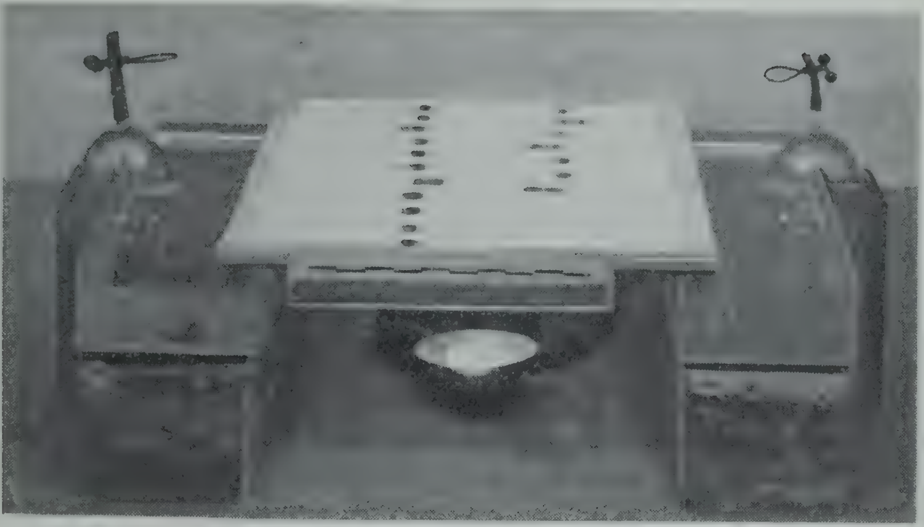

FIG. 1. Photograph of a broad, thin starch block resting on the edges of electrode vessels. Multiple human hemoglobin specimens are visible in the process of separation.

trough system (5, 6), and towels or thick filter paper at the ends drain off excess liquid.

The sample is usually applied by means of a thin-tipped pipette into a slit cut into the supporting medium. Regulation of the moisture content of the medium is important at this point, and the material should be just dry enough to preserve a slit. A block can be moistened with buffer or dried with a towel at any time to achieve the desired consistency. After insertion of the sample the slit is eliminated either by increasing the moisture content on either side or by simple manipulation with a spatula. The block is then thoroughly dried with a towel and the upper surface covered with polyethylene sheeting which extends to approximately one-half inch from either end. The application of the sheeting is a critical procedure and requires considerable experience and attention to details. For the starch system it can be easily applied so that it adheres to the surface as air is eliminated in a spreading operation starting at the site of application of the sample. If the medium is too moist it "melts" during this procedure, and in the case of some of the other media it has to be applied very lightly. Some success has been achieved without a cover in contact with the starch. The evaporation is considerable, particularly in a cold room, and an elevated cover requires an absorbing surface or another means of preventing droplets from falling back on the block. In general the polyethylene contact surface has been most satisfactory. Double thicknesses of cloth cut from laboratory towels serve as connections with the liquid in the electrode vessels. These rest over the ends of the block extending onto the polyethylene sheeting and are in turn covered with a strip of additional sheeting to prevent evaporation.

The voltage applied depends on the medium employed and its buffer content. Most experiments have been carried out in a cold room, but this is not essential. The voltage used is usually just below that which causes slight warming of the glass plate or an increase in current. The use of a cooling jacket in a Lucite plate is advantageous in some systems where the time of the experiment is a factor and short periods of high voltage are necessary. However, the separation is no better with the cooling system and it is definitely not essential. At the end of the experiment, the cloth contacts are removed and dry towels applied to soak up excess moisture before the plate is moved. This is particularly important with the supporting media which tend to "melt." The medium should appear relatively dry before sectioning for analysis is attempted.

Several methods have been employed to recover the separated material from the segmented supporting medium. The commonest procedure is to pack the segment in a small ground-glass filter, add buffer or other appropriate solution in two additions above the packed material, and apply suction. A volume of liquid equal to the volume of packed medium usually suffices to displace the desired material from the supporting medium. A second method is simply to shake up the segment in a volume of liquid sufficient to give a fluid suspension. Centrifugation or settling of the supporting medium with time leaves a clear liquid which contains a portion of the desired material. This is a rapid method and is useful for analysis of small samples to locate desired fractions. Further recovery can then be achieved by the filtration method. A disadvantage of this procedure is the dilution that occurs. A third method, which is slightly more cumbersome but results in very little dilution of the sample, utilizes the centrifuge to spin the liquid from the interstices of the supporting medium (7). The segment is inserted into plastic tubes with small holes at the bottom and a thin layer of packed ground filter paper covering these holes. This tube is in turn inserted into a glass tube and elevated slightly by means of a loose glass plug. Low speed centrifugation then causes the sample to be driven out of the segment into the bottom of the glass tube. A fourth procedure which has been used for the gels is to overlay the segments with buffer solution and apply an electric field which causes the desired material to migrate out of the solid supporting medium (8).

A closed-system procedure similar to that described above has given good results in a number of laboratories. It is extremely flexible and may be adapted to large-scale preparative work. Thicknesses greater than 1½ cm have caused irregularities due to temperature gradients, and as a result greater capacity has been achieved by increasing the width of the packed surface. Starch blocks 60 cm. in width have been used that permit as much as 30 cc. of serum to be applied in a long band approximately 2 cm. broad. The separation is just as good under these conditions as when a narrow block is used. These very broad blocks have additional advantages, and multiple specimens can be applied and compared directly in the same experiment. In the case of hemoglobin as many as 30 different samples have been applied in two rows on a single broad block (Fig. 1).

The limitations of these block or trough methods are considerable. The success of the separation is completely dependent on the technique and experience of the investigator. Elaborate attention to small details is necessary and the results achieved in different laboratories may be strikingly different. The empiricism of these methods is also in part due to variations in packing which are not readily controlled. Elimination of the slit used to apply the sample and the application of the cover are steps in the procedure that remain somewhat cumbersome. Perhaps the chief disadvantage is the difficulty in obtaining complete recovery of the sample applied. Slight losses occur by adherence to the top and bottom sheeting and in the segmentation and elution, as well as through adsorption to certain of the supporting media. However, the simplicity of the apparatus and the fact that separations as good or better than those obtained with any other method can be achieved have resulted in wide application.

B. Horizontal Half-Cylinder Method

This procedure is extremely simple and requires very little special equipment (9, 10). A cylindrical glass tube is cut in half, giving two curved troughs which serve as containers for the granular supporting media. Excess buffer is drained off from the ends with towels or heavy blotting paper. The sample is applied and covered with polyethylene sheeting in a manner similar to that described for the ordinary block system. The cylindrical troughs rest on electrode vessels, and contact is made through cloth wicks. Various sizes can be used, ranging from 2 to 5 cm. in diameter.

The advantage of the method is the ease with which a packed system can be arranged. It is particularly useful for laboratories where only a small amount of electrophoretic separation is done. Large-scale preparative work is difficult, but analytical experiments with relatively small amounts of material are conveniently carried out in this system. Determination of isoelectric points (10), utilizing multiple troughs containing buffers at different pH's connected to partitioned anode vessels, are readily made. The limitations of the procedure are primarily related to the relatively poor heat transfer and thus to the establishment of temperature gradients particularly where large cylinders are employed.

C. Vertical-Column Electrophoresis

Several workers have described methods of electrophoresis in vertical columns (3, 11–13), and the use of this type of procedure has spread considerably in recent years. The method developed by Porath (14) and Flodin and Kupke (15) in Tiselius' laboratory is most widely used. Various types of cellulose and starch have been primarily utilized for packing the column. Earlier procedures utilized a U-tube in order to balance the pressure in the column. However, the direct insertion of the column into a cylindrical vessel filled with buffer, which serves as one electrode chamber, has represented a marked improvement (16). This buffer chamber also acts as a cooling system for the column. It is in contact with the bottom of the column, while the top of the column through a side arm connects with the other electrode vessel. The buffer in the two electrode vessels is brought to the same level. Various stopcocks facilitate the insertion and removal of the column and its connections without disturbing the liquid-filled packed column. Both the insertion of the sample and the elution at the end of the experiment are conducted with the removable column alone, without disturbance from the buffer vessel system.

Progressive improvement in the arrangement of the column system has been achieved in Tiselius' laboratory, and the apparatus is now much more readily manipulated than were the earlier models. The usual column is 105 cm. in length and 3.4 cm. wide, but considerably larger types have been used. The possibility of applying as much as 500 cc. of serum at the top of a column has recently been realized. Preparative electrophoresis on a very large scale appears probable, although heating problems producing asymmetrical effects represent a serious limitation. Differences in heat transfer and associated differences in conductivity from the center of the column to the outer portion are almost impossible to control, particularly with columns of large diameter.

The greatest advantage of the column procedure is the ready recovery of material following electrophoretic separation. The use of a fraction collector to receive material as it flows from the column brings to electrophoresis the well-established principles of column chromatography. This advantage, however, is gained at some expense to the resolution. Following the completion of the electrophoretic separation in a column, some broadening of the bands always occurs as the material proceeds down the packed medium during the process of elution. This is readily visible with hemoglobin. As much as a twofold increase in the width of the color band can be seen to occur if a considerable portion of the column needs to be traversed during elution. Bands that have migrated electrophoretically to the bottom of the column are broadened considerably less. Thus the width and shape of a peak that is obtained by analyses of column fractions must be interpreted with these variations in mind.

Elution from the column also produces a tailing effect that was not present in the original electrophoretic separation. The degree of tailing is very dependent on the packing and elution procedures and may be variable in different experiments with the same material. Small amounts of protein remain trapped in various parts of the system immediately following passage of a large protein peak. This is more marked than in chromatography work because all material proceeds from the column with the column volume of liquid. Small inhomogeneities in the system produce exaggerated distortions. This effect proved disturbing to the present authors in their early work on column electrophoresis (3) and has not been entirely overcome by the elegant packing and elution methods of Flodin and Kupke (15). A curve for ribonuclease showing some tailing was illustrated in the latter publication. The authors state that it was not clear whether this was caused by adsorption of the ribonuclease to the column or by too rapid elution. Workers in the field have been frequently confronted with this problem, particularly when working with high concentrations of materials containing small amounts of more slowly moving contaminants.

Very recently Porath and assoc. have described an interesting modification of the column procedure whereby zones are remved from the column during electrophoresis by a buffer flow in the opposite direction to the electrophoretic migration (16a). This aids in eliminating displacement irregularities and permits the use of shorter columns.

Detection of colorless proteins during passage through the column has recently been carried out by Tiselius, utilizing ultraviolet absorption and visualization on a television screen. This appears to be one solution to an old problem in zone electrophoresis. Faintly colored bands are somewhat difficult to visualize in columns with curved walls. The observance of small traces of color in work with serum proteins in the open-block technique by means of bright transmitted light, particularly with the starch medium (Fig. 1), has always been an advantage of the latter procedure that is partially lost in the column method.

Despite these limitations and the relative complexity of the apparatus required, there is no question about many future applications of column electrophoresis. The development of new and better supporting media that permit very homogeneous packing will certainly bring this about. The possibility of reutilizing a single carefully packed column for many experiments, without disassembly, offers many advantages.

D. Continuous Flow Method

The principle of the procedure has been applied primarily to filter paper sheets with the liquid flowing through a broad paper sheet at right angles to the electric field. The method developed by Durrum *et al.* (17) has found the widest application, although a number of modifications are also used (18, 19). Other supporting media have been applied to this system primarily by Brattsten (20, 21, 22). Excellent separation of serum proteins on a large scale has been achieved employing starch, glass beads, and cellulose powder as the packed medium in a vertical rectangular chamber with electrodes at the sides, inlet channels at the top, and as many as 50 outlet tubes at the bottom. A pumping device regulates the hydrodynamic flow of electrolyte through the chamber. The apparatus used by Brattsten is very complex, but many of the pitfalls of continuous flow electrophoresis have been overcome. A regulating device (21) which controls the voltage as well as the hydrodynamic flow of liquid according to the current in the system has aided greatly in eliminating variations in the outlet position of substances under separation. The net displacement of the substance depends on the ratio of electric and hydrodynamic velocities, and it is extremely important to keep this ratio constant. The close correspondence of individual serum fractions with specific outlet tubes in different experiments on different days indicates that satisfactory control of this ratio has been achieved (21). An ingenious procedure for isolating the electrodes, employing cellophane and parchment membranes, has also aided in controlling the electroosmotic flow. A simplified apparatus has been described by Bradish and Smart (23) which eliminates multiple inflow tubes. However, the comparative effectiveness of this method remains to be determined. These authors also utilize this system for noncontinuous separation; a band of material is allowed to flow in, then separated by electrophoresis, and finally the fractions are isolated by hydrodynamic flow after separation.

The Brattsten apparatus has thus far not been used extensively in other laboratories mainly because of the intricate mechanical and electrical accessories that are required. However, it appears probable that with further refinements and commercial production, it will find wide usage. Certainly no other procedure available at present has the combined capacity and separating power obtainable with this apparatus.

E. Segmented-Sponge Method

The possibility of utilizing multiple sponge segments as support for electrophoretic separation has intrigued numerous workers. In general the method has been inferior to the granular media, particularly for fine resolution. However, some success has been achieved with sponge rubber (24), and the procedure is relatively easy to set in operation. Strips 7 mm. wide and 21 mm. long are cut from a ½-inch sheet of foam rubber (standard, flat stock, medium, U. S. Rubber Co.). These are placed in close contact with each other. One segment contains the sample to be separated, the remainder contains buffer. The main advantage of the method is the ease of obtaining samples by simply squeezing out the sponge segments following electrophoretic separation. Full recovery is obtained only after multiple extractions. Because of the large size of the pores in the sponge, convection is a considerable problem and results in substantial broadening of the bands.

F. Gel Electrophoresis

Many procedures for separation in gels have been described (6, 25–27). They differ primarily in the type of gel utilized, the concentration of the gel, and the presence or absence of filler substances. All of the currently used methods employ a horizontal trough or block system similar to that described for the granular media. Four main types of gels are being most widely used and will be described separately.

1. Simple Agar

The use of approximately 3% agar was first described by Gordon and his co-workers (6) and has become widely popularized through the work of Grabar and Williams (28). This type of gel is readily handled and can be stained by procedures similar to those used with filter paper. Excellent staining procedures for protein, lipid, and carbohydrate have been developed by Uriel and Grabar (29, 32). There appears to be very little adsorption of proteins in the path of migration as indicated by the two-dimensional experiments of Bussard and Perrin (25) with albumin and γ-globulin. The material to be separated is usually applied into a rectangular slit in the gel and is mixed with unsolidified gel prior to application. Some protein always remains adsorbed in the slit, and this is a disadvantage of the procedure. During electrophoresis the agar is either placed in a closed atmosphere or covered. Mineral oil, polyethylene sheeting, or a spray of polyvinyl chloride in methylene chloride (25), which hardens secondarily, have been used to avoid evaporation. Contact at the electrodes is made with filter paper strips. Some workers have prepared the gel on microscopic slides.

The method is primarily analytical, but isolation of separated fractions is possible. Faure and his associates (30) were able to recover various types of hemagglutinins by simply permitting the gel sections to stand in 2 or 3 volumes of isotonic saline followed by centrifugation. Others have employed freezing and thawing or liquefaction by means of a syringe followed by centrifugation. A preparative procedure that employs a somewhat different principle has been described by Kawerau (31). Here a semicontinuous system is employed and proteins that are electrophoretically and electroosmotically neutral remain in the hole of insertion of the specimen while the rest of the proteins migrate away into the agar.

2. Fluid Agar

A promising method of electrophoresis in a semisolid fluid film has recently been described by Ressler and co-workers (26, 33). Agar at only 0.13% concentration is used. A siliconized plate with glass borders glued onto the plate serves as the receptacle for the hot agar solution. The sample is applied by wetting a thin piece of filter paper with protein solution and placing this on the film perpendicular to the path of the current. Contact with electrode vessels is made with filter paper wicks, and the system is placed in a closed chamber with blotting paper on the upper surface to prevent a backflow of liquid. After electrophoresis, the glass plate with the film is removed and placed over infrared heating

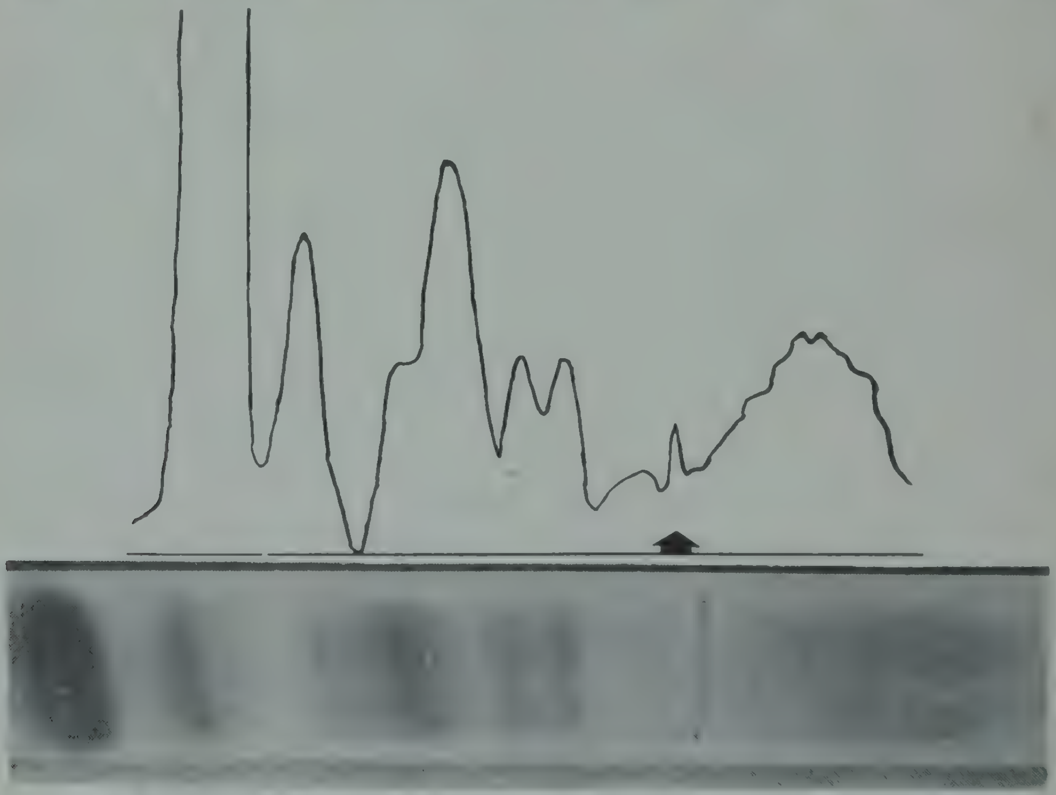

FIG. 2. Electrophoresis of human serum in semisolid agar medium. The stained proteins are visible on the dried agar film. Barb. buffer, pH 8.6; $\Gamma/2$ 0.03 [Ressler and Zak (26)].

lamps. The agar dries to the plate, which can then be dipped into staining solutions and colored in the usual manner. Densitometric analyses of the colored bands on the glass plates can be carried out directly.

The separation achieved appears to be equal or better than with the higher agar concentrations (Fig. 2). Prolonged separation shows evidence of considerable subfractionation of serum proteins. The low concentration of agar gives considerably less electroosmotic backflow. A disadvantage of the procedure appears to be the difficulty in controlling the rigidity of the medium; time of standing causes alterations, and mobilities show resultant variation.

3. Starch Gels (Low Concentration)

Bernfeld and Nisselbaum (8) have recently described a procedure of gel electrophoresis that utilizes a mixture of 3% corn starch, 3% Hyflo-Super Cel, and 1.5% amylose. The addition of amylose permits gel formation at a lower starch concentration than would otherwise be necessary to give a solid gel. The Hyflo-Super Cel simply improves the elasticity and mechanical resistance of the product. These authors attempted to avoid any hindrance to the free electrophoretic migration of proteins and found it necessary to use a low concentration of starch. The addition of amylose creates additional problems because of the difficulty in removing it from the isolated protein fraction. However, good separation is possible by this procedure, and the relative mobilities of serum proteins are close to those obtained in free solution. The electroosmotic flow is extremely low, even less than with granular starch, and far less than with agar gels. A preparative adaptation of the method has also been described (8) which permits the isolation of relatively large amounts of serum proteins. Recovery of proteins appears excellent.

4. Starch Gels (High Concentration)

The method of Smithies (27) of starch gel electrophoresis is finding wide application because of its unique separatory power for the serum proteins. Soluble potato starch at approximately 15% concentration in a weak borate buffer is employed. This gives a gel of considerable strength that can be handled particularly for staining the separated proteins. The sample is applied in a slit in the gel either by means of the insertion of filter paper containing the sample or through the application to the slit of granular starch containing the sample. The proteins are stained with Amido Black (27) in a manner similar to that used with filter paper. Because of the low ionic strength of the borate buffer considerable variation in the potential gradient over the gel is encountered. This is particularly true in the vicinity of albumin when serum proteins are separated. Difficulties in standardization of the procedure would be expected and this represents one of the limitations of the method. Small variations in the type of starch and the time and conditions of hydrolysis lead to differences in resolution.

The outstanding attribute of the procedure is the fact that serum proteins separate into as many as twelve components which can be visualized as sharp lines in the stained gels. The exact reason for this increased resolution is not entirely clear. Multiple factors probably play a role. First, sharpening of the sample band probably occurs at the site of origin as the proteins are slowed on entry into the gel. This gives an extremely narrow origin—narrower than those used by other workers with other media. Second, the weak borate buffer may produce specific effects, particularly in a potential gradient that is affected by variations in protein concentration. The borate ions may also complex with some of the carbohydrate proteins, thus producing further separation. Third, the high concentration of starch in the gel probably produces a pore size that is not too different in molecular dimensions from that of the proteins being separated. A sieving effect on the basis of size and shape appears to occur. Evidence for the latter effect has accumulated. Experiments conducted with a high molecular weight α_2-protein supplied to Smithies by the authors indicate that this fraction is specifically retarded in comparison with other α_2-proteins (34). Fibrinogen does not migrate freely in the starch gel. Additional indirect evidence also supports the molecular sieving hypothesis although it remains to be clearly proven.

Albumin and γ-globulin behave peculiarly in the starch gel system, and increased resolution of these proteins has not been achieved thus far. The location of the site of application, which contains some nonmigrating proteins, in the γ-globulin region hampers clear interpretation of the stained pattern of the whole serum and represents another limitation. The application of the method to a two-dimensional system appears very promising. Smithies and Poulik (35) have recently described a procedure by which a filter paper strip containing proteins separated in the usual manner is inserted into the starch gel at right angles to the electric field. Secondary separation of the serum fractions then occurs in the gel. The further resolution of the α_2-globulins into six subfractions represents a striking finding (Fig. 3). Very little preparative work has thus far been attempted

FIG. 3. Starch gel electrophoresis pattern of human α_2-proteins. This preparation, which showed a single peak by conventional electrophoretic methods, separated into eight subfractions. (Experiment of Dr. A. G. Bearn.)

with this gel system, and it remains problematical whether it can be adapted to large-scale isolation work. The fine detail achieved in analytical experiments is partly the result of slicing the gel strip through the middle, thus exposing the inner portion for visualization through staining. The separation is not completely homogeneous throughout the gel strip and surface distortions are common. Such variations would cause difficulties in preparative work where the gel would have to be segmented in a complete cross section without direct visualization of the distorted areas.

III. Comparison of Different Supporting Media

Most of the supporting media that have been applied to zone electrophoresis can be used either in the column or open-block form. This is not true of the gels, however. It has not been possible to utilize the principles of column electrophoresis for this medium thus far because of the great difficulty in eluting the separated fractions by hydrostatic pressure applied to the top of a gel column. In most instances the gel disintegrates at pressures where liquid can be forced out. Some new and different principle will have to be employed; possibly electroosmotic liquid flow may be harnessed for elution purposes.

A. Powdered Cellulose

Recently Flodin and Kupke (15) have described a powdered cellulose that appears to have specific advantages for column electrophoresis. This is obtained through the hydrolysis of cotton fibers by refluxing in the presence of strong acid. Many of the fibrous properties of cellulose materials are lost and the material settles as a fine powder for homogeneous packing in a column. Basic proteins which have presented many problems when separated in ordinary cellulose media, show very little tailing. Ultraviolet-absorbing contaminants picked up by albumin also appear to be very much reduced in this medium.

Cellulose in general has not lent itself well to horizontal-block electrophoresis although some satisfactory results have been obtained in a number of laboratories (36, 37). This has been due in the past to difficulties in obtaining a sharp origin and segmenting the block at the end of the experiment. The fibrous quality of the wet cellulose slab makes sharp slicing almost impossible and a tearing procedure is required. The new cellulose medium of Flodin and Kupke shows decided advantages in these respects although difficulties have still been encountered in the authors' laboratory.

B. Granular Starch

The most readily handled supporting medium for trough or open-block electrophoresis remains the granular starch system. The packing can be made very homogeneous and there is very little tendency for the block to "melt" with handling. Sharp bands can be applied at the origin, and segmentation at the end of the experiment is easily achieved. Removal of fractions from the starch is quite simple by the various procedures described earlier in this chapter. Contaminating materials from the starch can be reduced to a minimum by preliminary settling procedures which eliminate soluble and small insoluble particles (5). All other media tested in the authors' laboratory show practical disadvantages when compared to granular starch for small-scale preparative electrophoresis by the open-block method.

Despite these qualities the starch medium suffers from many disadvantages. It works well for most large-sized proteins, but most peptides and proteins below 30,000 in molecular weight do not migrate freely. This appears to be primarily due to penetration of the smaller molecules into the swollen starch granules. The serum proteins, for example, do not penetrate these granules and their space in the packed medium is much smaller than the buffer ion space. Oxytocin and vasopressin on the other hand, two smaller peptides, show tailing effects as they migrate through the starch, and their space is greater than that occupied by serum albumin. As a result it is generally inadvisable to employ this medium for low molecular weight materials.

A second serious limitation of the granular starch system is the problem of trace contamination derived from the medium itself. Small amounts of soluble carbohydrate, phosphorus-containing compounds, ninhydrin-positive material, and ultraviolet-absorbing substances may cause difficulties in analyzing the isolated fractions that have been separated. Determination of the carbohydrate content of a protein isolated from the starch is difficult, although hexosamine analyses are perfectly feasible (38). Pyrogenic materials and substances causing red blood cell hemolysis have also been encountered in working with crude starch. Many of these problems can be overcome by utilizing the settling procedure described above for purifying the starch. This is a somewhat tedious method, and despite its distinct value for many experiments the crude starch is still employed most frequently. Problems of recovery have also been encountered in working with the starch system. Some proteins such as hemoglobin show 100% recovery; others such as serum albumin, and γ-globulin are in the 80–90% range. It appears to vary with the protein under study. Serum lipoproteins offer the greatest difficulties, but this is probably due to the affinity of many lipid materials for starch grains, particularly in the presence of polar solvents. Another disadvantage of the starch system is the difficulty encountered when the same starch is used more than once. This is particularly important in column work where it is advantageous to utilize a well-packed column for multiple experiments. Weak buffers also present difficulties because of ion exchange properties of the starch grains; with alkaline buffers there is often a pH fall, particularly if thorough equilibration is not permitted to occur.

C. Polyvinyl Chloride Resins

Polyvinyl chloride (Geon Number 426, Goodrich Rubber Company) has been found to be a useful replacement for the starch system in those instances where the difficulties described above prohibited the use of granular starch (39). It can be used satisfactorily in the open-block procedure although it is somewhat more difficult to handle than starch. A sharp origin can be applied, and segmentation after separation and isolation is readily achieved. Recoveries of serum proteins have been excellent. Amino acids, carbohydrates, peptides, and small as well as large proteins have given good results. The main disadvantage of the polyvinyl chloride medium is a considerable electroosmotic backflow causing a cathodal shift of all components. Possibly for this reason it is important to thoroughly dialyze and equilibrate proteins with buffer prior to separation. Undialyzed whole serum gives fair results with the starch medium but very poor results with polyvinyl chloride. The tendency of the polyvinyl block to "melt" under pressure is another disadvantage, but one which can be avoided with experience. Only a few of the many polyvinyl resins have thus far been explored, and it is very possible that a better medium of this type will be found. It is necessary that the medium be wetted by aqueous solvents, and this usually means introduction of acid groups which cause high electroosmotic flow and perhaps other undesirable features. The use of these resins in column electrophoresis has not yet been investigated.

Other media, such as various types of glass beads and other silicate materials, although finding application in earlier work, have now been supplanted by the polyvinyl chloride and starch media.

D. Gels

Agar and starch gels have thus far been used primarily as analytical tools although the method of Bernfeld and Nisselbaum (8) shows promise as a large-scale preparative method. Problems of soluble contaminants from the medium which are difficult to remove represent a serious limitation. Traces of carbohydrate and other substances persist following isolation from agar or soluble starch. The chemistry of agar is poorly understood, but most evidence suggests that it is a galactan ethereal sulfate. The presence of one ethereal sulfate to each galactose unit makes agar a strongly acidic substance, and this property is probably one of the most serious disadvantages. The electroosmotic backflow is considerable. Although this alone does not appear to hamper resolution, as is commonly thought, the shift does produce some tailing and makes interpretations of net charge more difficult. There is also some evidence that anion-binding may occur during the separation in agar. Very peculiar results have been obtained with serum lipoproteins (32). Their relative mobility is markedly increased and the best explanation of this change is that acidic substances have been picked up from the agar. The effects of soluble contaminating materials in the starch gels has not as yet been investigated. Possibly the use of very low concentrations of gels in the semisolid system described above will circumvent some of these difficulties.

Adsorption appears to be a very minor problem with the agar and starch gels, and as a result they show considerable promise for two-dimensional electrophoretic work (25). The occurrence of the site of application in the midst of the separated proteins is a disadvantage of both the agar and the starch gel systems particularly since some of the serum proteins are trapped here and fail to penetrate the main gel block. Certain macroglobulins appear to be retained selectively at the origin (40) although this is in part due to the large size of these proteins; reduced solubility also appears to play a role, particularly with agar.

It now appears that the gels offer advantages over all other supporting media for analytical separation. Extreme sharpness of the line of origin and minimum distortion of the various bands on separation make for the fine resolution achieved by this method. The use of semisolid gels at low agar or starch concentrations shows considerable promise mainly because this eliminates some of the undesirable features of the high concentration gels. Other types of gels also will undoubtedly be utilized. Cellulose acetate which is replacing filter paper in some areas is almost a gel and has the advantage over filter paper of transparency (41).

The molecular sieving effect of the high concentration starch gels has thus far been applied primarily to resolving the serum proteins. However, widespread application of this unique property will undoubtedly develop. Studies of the homogeneity of highly purified proteins are particularly suited for this gel system. Crystalline ribonuclease, for example, clearly shows three bands in the starch gel (42), and these correspond to fractions isolated by Moore and Stein by ion exchange chromatography. The method remains a powerful analytical tool, and preparative utilization of its high resolving power awaits further developments in technique.

IV. Specific Application

A. Immunoelectrophoresis

The combined use of electrophoresis and secondary reaction with antiserum in agar has proven a valuable tool in immunology for the study and characterization of various antigen-antibody systems (28, 40, 43). Figure 4 illustrates the multiple bands obtained for human serum after separation and diffusion against a horse antiserum made to whole human serum. This method permits detection of at least 18 different components in human serum. Purified proteins from serum studied in this way have often revealed contaminants that were not apparent by other methods. The procedure can also be reversed and the antiserum separated electrophoretically with secondary diffusion against antigen (44). The multiplicity of components in antigen-antibody precipitates has been demonstrated by immunoelectrophoresis (45). Absence of specific proteins in human sera in disease has also been indicated by this procedure (46, 47). Absorption of the antiserum with serum deficient in certain components has revealed these components strikingly in reactions between normal serum and the absorbed antiserum. Abnormal serum proteins, such as the myeloma group and the macroglobulins, have been successfully studied (40, 48). Recently immunoelectrophoresis has been applied to the problem of characterization of separate antigenic groups within a single protein (49). Hydrolysis of the

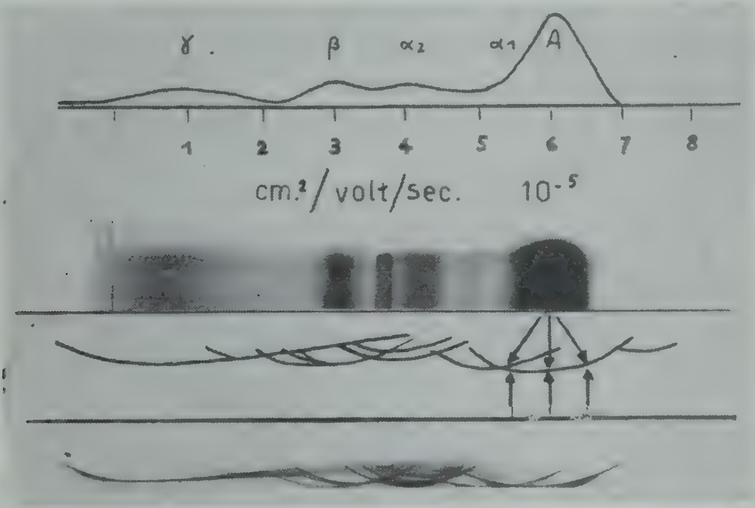

FIG. 4. Immunoelectrophoresis pattern of human serum. The lines in the lower half of the figure represent antigen-antibody precipitates obtained by diffusion of the separated proteins against horse antihuman serum antibodies. (Grabar, P., *Actes du Coll. sur la Diff. Mont.* June, 1955.)

protein followed by agar electrophoresis and diffusion against antiserum toward the whole protein has revealed a number of different peptide antigens. This application, which shows considerable promise, will undoubtedly be used further in the future.

B. Haptoglobin Separation

The major application of the starch gel electrophoresis system thus far has been to the problem of the different genetic groups of haptoglobins which occur in normal serum of humans and a variety of animals. The haptoglobins are characterized by their striking ability to bind hemoglobin. Three haptoglobin types have been observed in human serum (27, 50) which are recognized by the fine-band pattern that one obtains by the starch gel system (Fig. 5). These have been designated haptoglobin 1-1, haptoglobin 2-1, and haptoglobin 2-2 and are present at approximately a 1:2:1 ratio. Variations in this ratio are of considerable interest to human geneticists, and differences have already been encountered in South African populations (51). The various haptoglobins appear to differ in size (52), and it is primarily the selective filtration on the basis of size and shape in the starch gel system that accounts for their resolution. However, these proteins also differ in charge, and separation of the three types has been achieved in the authors' laboratory by simple granular starch electrophoresis. This can be detected by observing the mobilities of the red-colored haptoglobin-hemoglobin complexes.

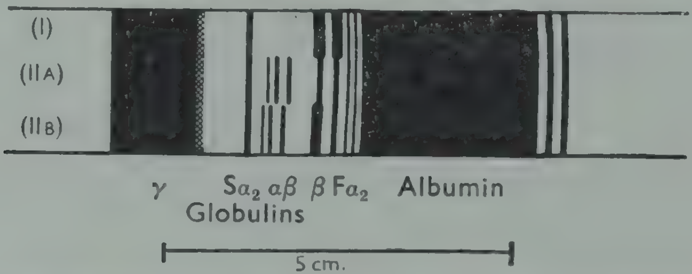

FIG. 5. Starch gel electrophoresis showing the different bands obtained in the three genetic types of normal human serum. The absence of bands in the $\alpha\beta$ region of (I) is illustrated. [O. Smithies (27).]

C. Separation of Normal and Abnormal Hemoglobins

The white background of the granular starch medium has made it possible to detect minor hemoglobin components in normal human blood that were not observed by other electrophoretic procedures (53). One of these, A_2-hemoglobin (54), has been studied in considerable detail and shows a very constant level of approximately 2.6% in normal individuals

It has special significance in thalassemia where it is constantly elevated, with levels reaching as high as 12%. The abnormal human hemoglobins are also advantageously detected and quantitated by this procedure (Fig. 6).

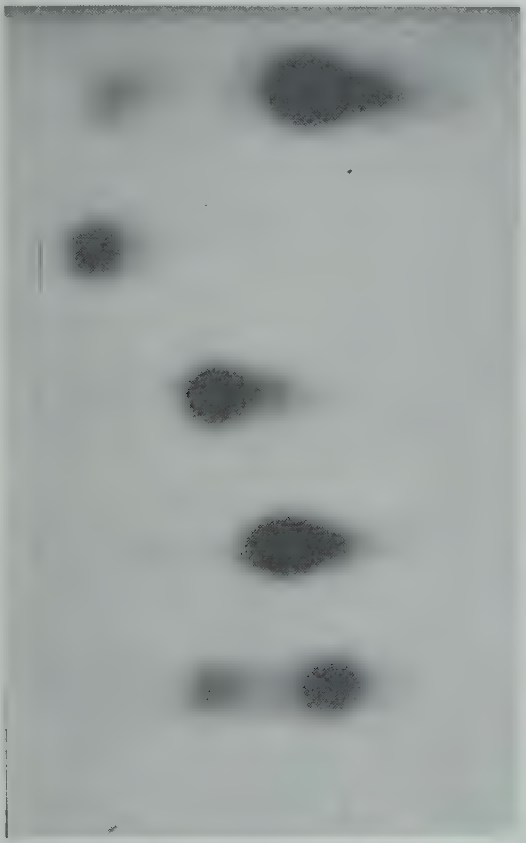

FIG. 6. Separation of human hemoglobins on a thin starch slab. Proceeding from top to bottom these are: normal A_2 and A hemoglobins, C hemoglobin, S hemoglobin, fetal hemoglobin, S and A hemoglobins. The line at the left is the site of application.

Recently, unique fractionation of hemoglobins has been obtained by Zuelzer and his associates (55), utilizing ordinary agar electrophoresis at pH 6.5. Apparently some chromatographic separation is superimposed on the electrophoretic migration and a number of components are observed which do not separate from the main hemoglobin by other methods of electrophoresis. This is particularly true of fetal hemoglobin which shows a marked cathodal migration which clearly separates it from other hemoglobins.

D. Separation of Antibodies

Considerable fractionation of γ-globulin can be achieved by simple procedures of zone electrophoresis. Rerunning of the various fractions making up the broad γ-globulin peaks observed in starch-block electrophoresis indicates that each has a slightly different mean mobility (39), and there remains little doubt that the total γ-globulin represents a vast spectrum of proteins with similar but slightly different mobilities. This was evident from two-dimensional electrophoresis experiments where this group of proteins failed to migrate as a round spot but separated into a diagonal line (25, 56).

Numerous antibodies have now been separated within the γ-globulin fraction by means of starch-block electrophoresis. This method supplies just sufficient antibody for the usual procedures of quantitation and therefore is unusually suitable for such work. Figure 7 illustrates the separation

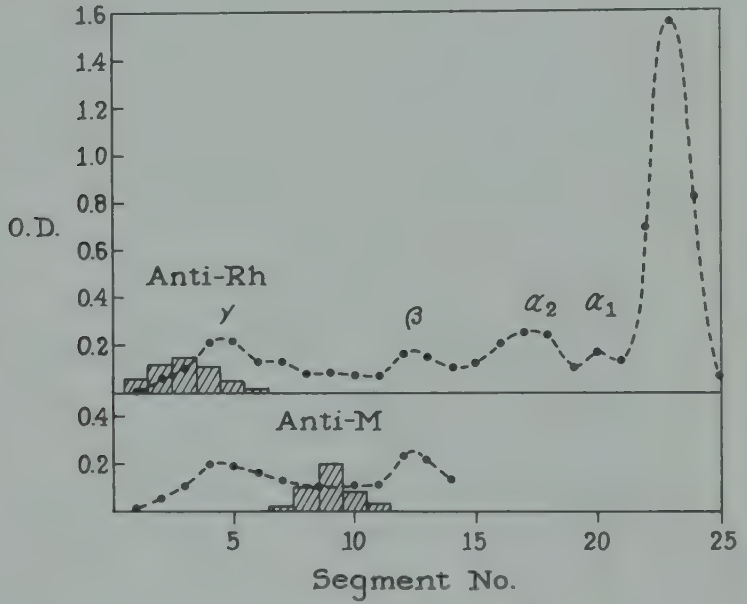

FIG. 7. Localization of two types of antibodies in different regions of the γ-globulin in two sera separated simultaneously on the same starch block. Barb. buffer, ph 8.6, $\Gamma/2$ 0.05.

of two types of antibodies studied in the authors' laboratory. The incomplete Rh antibodies migrate as slow γ-globulins while the anti-M agglutinins are fast γ-globulins. This is an extreme situation and usually the different antibodies overlap considerably with only the peak concentration showing separation. Application to the problem of diphtheria antibodies (57), skin-sensitizing antibodies (7, 58), viral antibodies (59), and hemolytic agglutinins (60) represent a few of the examples where this method has been used for antibody separation.

E. Homogeneity Studies

Probably the greatest specific value of the various methods of zone electrophoresis either in open blocks or in columns lies in the fact that sufficient material is obtained in each fraction to permit several types of analyses to be made simultaneously. This makes it possible to plot curves and thus observe correspondence or lack of correspondence between these measurements. Thus various activities can be correlated with a certain protein peak, and if such an association holds over the entire curve of the peak, particularly after electrophoresis at more than one pH, strong evidence for association between the two is obtained. It is in this type of work that the zone methods, particularly those where isolation of fractions is readily achieved, hold great advantages over free-solution electrophoresis. Examples are numerous where purified proteins have shown a single protein peak by both free-solution and zone electrophoresis. However, when activity measurements are made on the zone electrophoretic fractions, the activity, thought to be associated with the protein peak, is found migrating separately at a point where little or no protein is detectable. Enzymatic activity, prosthetic groups, lipids, carbohydrates, and nucleic acids represent a few of the additional parameters that can be studied in this way for specific proteins. When utilized in this way, the zone methods offer a powerful and not fully appreciated tool for determining the purity of protein preparation.

V. Theoretical Complications Due to the Supporting Medium

A. General

The purpose of the supporting medium when used in zone electrophoresis is to minimize convection due to hydrostatic pressure differences between solute-containing and solute-free regions. As such, it serves a useful purpose, but only at the expense of occupying space and complicating the current path. In order to call attention to this, the requisite parameters describing the effects of the porous medium will be expressed in such a manner that they vanish as the fraction of the total volume which is occupied by the support goes to zero. The factors to be described may not be the only ones that need be considered in the interpretation of all electrokinetic experiments, but they must be applied before others are invoked or quantitated. Thus, in starch gel electrophoresis the additional factor of molecular sieving must be considered. Similarly if the support contributes chromatographically to the migration as in the case of agar at acid pH, then geometrical factors alone will not suffice to describe the velocity of the components. The complete block or strip of the supporting medium is like the membrane or porous plug employed in electrokinetic experiments to ascertain properties of the solid. Whereas the over-all phenomenological understanding of such a system is valuable and will be considered first, it is secondary to the goal of zone electrophoresis—the separation of solutes from the initial zone. In effect, attention must ultimately be focused on the processes taking place *inside* of the membrane or plug.

In free electrophoresis the current path length is not the shortest distance between the ends of the U-tube of the Tiselius cell. Similarly, the average current path in supporting media may not be simply the container length. The conducting network of interconnected channels has made it difficult to visualize, let alone calculate, this effective path. None the less, theoretical investigations of inhomogeneous conductors date back to Clerk Maxwell, and experimental studies to Lord Rayleigh (61).

In each field where porous media are involved, investigators have introduced terminology peculiar to their own discipline. Each group has rediscovered or reworked the basic statistical parameters needed in the geometrical description of porous media. Thus, for example, the following related terms have been used: porosity, tortuosity, formation factor, conductance capacity, pore factor, effective cross section for conductance, void volume, fraction of buffer displaced, permeability, hydraulic radius, column volume, absorbance, wetness, and obstructive factor.

Buried in the above list are three basic parameters. One has to do with how much of the total volume is solid and how much fluid, the second has to do with the structure of the solid as regards its dispersal of the fluid into tortuous channels, and the third has to do with the surface area of the solid which is in contact with the fluid. The first two will be considered in the next several sections. The third will be considered in the section on hydraulic flow where it will be seen that the three are not sufficient and a fourth parameter is needed. With a direct-current electrical field and a pressure gradient, there will be both current and fluid flow. The cross term in the interaction of the two fields is the electrokinetic effect and requires the introduction of a fifth parameter, the zeta potential, for its description. This is not only a property of the supporting surface, but also of the impregnating fluid. After discussion of electrokinetics, the applicability of all these factors to the determination of the mobility of certain solutes from zone displacements is considered.

B. Supporting-Medium Content Parameter (Support Content S)

The basis for the geometrical definition of the parameter describing how much solid supporting medium is present is indicated in Fig. 8. Consider a unit cube of the medium of side l as in (a) with volume =

In (b), (c), and (d) are shown various arrangements of the solid, indicated by diagonal lines. In (b) there is a single straight pore of cross-sectional area q'' and volume $v' = lq''$. In (c) the pore is diagonal. It is drawn with the same area q'' on the left and right faces measured perpendicular to the edge of the cube. It can be seen by mentally sliding the lower right shaded triangle up to close the pore, that the volume v' of this pore is equal to that shown in (b). In (d) each of the two pores is also of volume v', being the same diagonal length as in (c), merely folded over to allow two in the same space as the one in (c). Thus the amount of support is the same for (b) and (c) but less for (d). To quantitate this, define the supporting-medium content parameter (abbreviated to "support content") S as the ratio of the volume of solid to the total volume. If this is calculated from geometry, then

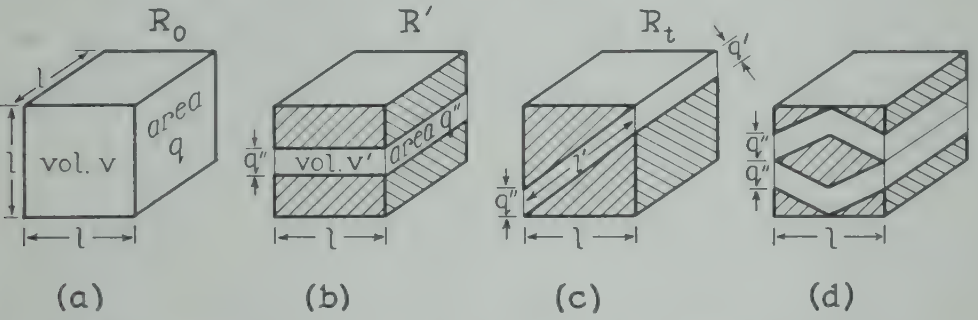

FIG. 8. Schematic distribution of solid and fluid in a supporting medium indicating geometrical basis for support content S and structure coefficient T parameters.

Diagonal lines represent solid; v, total volume; v', fluid volume; l, container length; l', pore length; q'', cross-sectional area of fluid perpendicular to l; q', cross-sectional area of fluid perpendicular to l'.

S is equal for (b) and (c), but diminished in (d).

T is equal for (c) and (d), but diminished in (b).

R represents resistance when cube is considered as a conductivity cell, electrodes of area q on left and right hand faces.

$$S \equiv 1 - v'/v \tag{1}$$

In Fig. 8, $S = 1 - q''/l^2$ for the arrangements of (b) and (c), but in (d) $S = 1 - 2q''/l^2$. The ratio v'/v, frequently called the "porosity" of a porous medium, has been recognized as a valuable parameter for almost a century.

C. Path-Length Parameter (Structure Coefficient T)

Also indicated in Fig. 8 is the length l' inside the pore. In the arrangement of (c) l' is greater than l and hence greater than the pore length of (b). In (d) l' is the same as for (c). To quantitate this notion of path length being longer than the container length (56, 62), define the path-length parameter or "structure coefficient" T as

$$T \equiv (l'/l)^2 - 1 \tag{2}$$

In Fig. 8 (b) $T = 0$, but for the arrangements of (c) and (d) it is equal to unity.

It should be noted that $(l'/l)^2$ itself has been called tortuosity (63). The two terms porosity and tortuosity are not used here because as words they do not put the emphasis on the supporting medium, nor are they sufficiently different mnemonically. The reason for the square will become apparent when the operational definition is considered.

D. Operational Definition of S

Some supporting media, and perhaps most, swell. This means that there is immobile fluid inside the grains of the medium which contributes to the effective volume of the support and may or may not contribute to the current path. In this case a distinction is made between the wetness W and the support content S as follows:

$$W \equiv \frac{v_{\text{trapped}} + v_{\text{displaceable}}}{v_{\text{total}}} = \frac{v_{\text{fluid}}}{v_{\text{total}}} \tag{3}$$

$$S \equiv \frac{v_{\text{total}} - v_{\text{displaceable}}}{v_{\text{total}}} \tag{4}$$

The relationship between S and W is obtained by eliminating $v_{\text{displaceable}}$, the volume of fluid which can be displaced from a column packed to the same wetness as used in the electrophoresis:

$$S = (1 - W) + (v_{\text{trapped}}/v_{\text{total}}) \tag{5}$$

Both McDonald *et al.* (64) and Crawford and Edward (65) have considered the formula for calculating W from density and weight measurements:

$$W = A/(A + 1/d_s) \tag{6}$$

where A is the volume of fluid in milliliters present in 1 gm. of dry support, called "absorbance," and d_s is the density of the dry support in grams per milliliter. In addition, the latter authors have given relationships for calculating the effect of adsorption of water and swelling to yield $(1 - S)$ nonoperationally for cellulose fibers.

The conditions for the equivalence of equations (1) and (4) need no special mention.

E. Operational Definition of T

In order to avoid complications due to surface conductivity which will be defined in Section V, H, a suitable high-conductivity fluid such as 0.1–1 M KCl is used to impregnate a nonswelling, insulating supporting medium for the following alternating-current resistance measurement. The entire porous medium with its fluid can be considered a conductivity cell or an actual cell can be constructed. Schematically, the arrangement can be indicated as in Fig. 8, where the left and right faces can be considered the electrodes. Its resistance R_t (Fig. 8c) will certainly be higher than the resistance R_0 (Fig. 8a) of the cell filled with fluid alone. But rather than compare R_t with R_0, we wish to compare R_t with the calculated resistance R' (Fig. 8b) that the displaceable fluid would have if it occupied the conductivity cell of the same length, but of course not filling it. The operational definition of the structure coefficient T is

$$T \equiv R_t/R' - 1 \tag{7}$$

It is the structure coefficient T which must be known before a mobility can be calculated from zone electrophoresis. However, its measurement is difficult since equation (7) is not universally applicable to all media. Application of a relative method is given in Section V, J. The failure of other processes to yield T will be considered in Section V, G and H. It must now be shown that equation (7) gives the same value for T as does the geometrical definition, equation (2). This is ultimately an experimental task. The reasonableness of the equivalence, however, can be seen by rearranging equation (7), using Ohm's law, as follows.

In general, for a solid, electrolyte, or porous medium the current I will be proportional to the impressed voltage E if

(a) High-conductivity fluid is employed to diminish electrokinetic effects (to be discussed in Section V, H).

(b) The frequency of alternating current is not too low for polarization effects at the electrodes (66), nor too high for the Wien effect (67) or the unexplained effect of Goring *et al.* (68) with swelling media to be involved.

(c) The diameter of the largest circle inscribed in the cross section is greater than 25 times the largest diameter of the particles when porous media are used in the conductivity cell (69).

Then

$$E/I \equiv R \tag{8}$$

is the over-all statement of Ohm's law, defining the resistance R. It is convenient to introduce for the conductivity cell of volume $v = lq$ the terms

$$e \equiv E/l \tag{9}$$

as the "voltage gradient" or "macroscopic field strength," and

$$i \equiv I/q \tag{10}$$

as the "macroscopic current density." Inserting these in equation (8) gives

$$\frac{i}{e} = \left(\frac{l}{q}\right)\left(\frac{1}{R}\right) = \left(\frac{l^2}{v}\right)\left(\frac{1}{R}\right) \equiv \kappa \tag{11}$$

where κ, defined hereby, is called the "specific conductivity" because it has been found to depend on the contents of the cell and not its dimensions. This equation (11) is the specific statement of Ohm's law.

If the contents of the cell are the free solution, denoted by a subscript zero, the equation (11) yields (Fig. 8a)

$$\kappa_0 = \left(\frac{l^2}{v}\right)\left(\frac{1}{R_0}\right) \tag{12}$$

as the "free-solution conductivity" or "conductivity in bulk" of the impregnating fluid.* Similarly, if the cell is partly filled by the volume v' of the displaceable impregnating fluid, then (Fig. 8b)

$$R' = \left(\frac{1}{\kappa_0}\right)\left(\frac{l^2}{v'}\right) = R_0\left(\frac{1}{1-S}\right) \tag{13}$$

where equations (1) and (12) have been used to obtain the right-hand side. When this same volume v' of fluid is in the porous medium it must have some average current path length, l'_c. Hence from equation (11) its resistance is describable as (Fig. 8c)

$$R_t = \left(\frac{1}{\kappa_0}\right)\left(\frac{l_c'^2}{v'}\right) = R_0\left(\frac{l'_c}{l}\right)^2\left(\frac{1}{1-S}\right) \tag{14}$$

where again equations (1) and (12) have been used (70, 71). In terms of R_0 and R_t, the operational definition of T, equation (7) becomes from equation (13):

$$T = R_t(1-S)/R_0 - 1 \tag{15}$$

and by further substitution of equation (14):

$$T = (l'_c/l)^2 - 1 \tag{16}$$

This is the same as equation (2) if it be assumed that the average of the actual current paths l'_c is the same as the statistical average of the geometrical paths l' (56). This is an unreasonable assumption for either the case of swelling or noninsulating media in which part of the current path penetrates individual grains. However, as will be seen in Section V, F below, where Fig. 9 is described, the relationship has been verified for various packing of spheres and is here assumed to be generally valid for other shapes of grains, too, if nonswelling and insulating. For these cases substitution of equations (12), and (15) gives in Ohm's law, equation (11), for $R = R_t$:

---

* Using the cell as a relative instrument it is convenient to introduce the term "cell constant" J_0 as $J_0 \equiv \kappa_0 R_0 = l^2/v$. Then an unknown's specific conductivity is calculated from the measured resistance R as $\kappa = J_0/R$.

$$\frac{i}{e} = \kappa = \kappa_0 \left(\frac{R_0}{R_t}\right) = \kappa_0 \left(\frac{1 - S}{1 + T}\right) \tag{17}$$

for the "apparent conductivity" where the impregnating high conductivity electrolyte is of bulk conductivity κ_0.

An alternative expression from equations (1) and (14),

$$F \equiv \frac{R_t}{R_0} = \frac{1 + T}{1 - S} = \left(\frac{l'}{l}\right)^2 \left(\frac{v}{v'}\right) \tag{18}$$

has been called the "formation factor" F of sands, shales, and porous rock (69, 72). It thus depends upon both the fraction of solid present as well as its structure. This conversion of F or R_t to effective length l' has been the source of considerable controversy, mostly as a result of confusion of the various areas involved (56, 69, 71, 73), or reluctance to ascribe effects to increased path length at all (64, 74). In particular in Fig. 8 are shown three choices q, q', and q''. If q', the area perpendicular to the pore current path l', is used in the area form of equation (14), then equation (18) results.*

F. Theoretical and Experimental Relationship between S and T

The special arrangements of Fig. 8 were chosen to show that, in general, S and T are independent parameters describing the geometry of the fluid space (75). In the complicated maze of pores in actual supporting media, S and T take on statistical meaning representing an averaging over all possible paths (76–80). It would then be anticipated that for a given shape of particle a change in the wetness would result in a change in the length of the current path. Hence T is a function of S for a given grain shape. Because of the mathematical difficulties, theoretical computations have been limited to various types of packing of unconsolidated spheres (78). The term unconsolidated means that there is no cementing or bridging between the grains or particles. Clerk Maxwell's relation was derived for very small values of S, the spheres being embedded in a conducting medium and having no points of contact (77). Slawinski's equation (ref. 76) is for spheres in contact, and is closely fit by the data of Wyllie and Gregory (69, 73). Part of their data from a complete experimental study have been recalculated according to equations (1) and (15) and are shown in Fig. 9. The labels *sphere*, *bead*, *disk*, etc. refer to packings of ingeniously made, nonswelling insulating particles of the named shapes.

---

* Equation (9) and the first unnumbered one below it in reference (56) used q'' instead of q' and therefore require some revision.

As might be anticipated, this Fig. 9 indicates that the more angular the particles, the greater is the structure coefficient. Also, if there is consolidation, then there can be a large increase in the average current path length, with little change in fluid space. But regardless of the kind of particles that are used in the supporting medium, the smaller the support content *S* (greater wetness), the smaller the structure coefficient *T*.

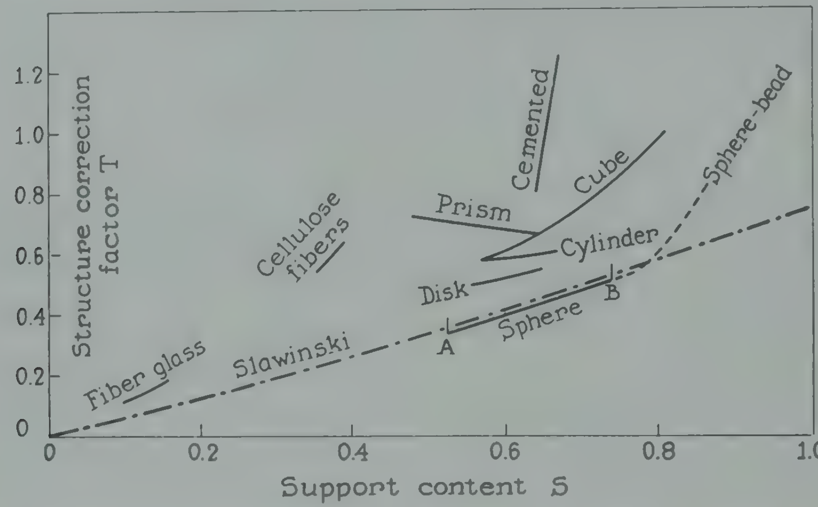

FIG. 9. Relationship between *S* and *T*. Experimental values indicated by solid lines for media composed of grains of the shapes indicated by Wyllie and Gregory (69, 73). Fibrous filter paper values from Crawford and Edward (65). Theoretical curve of Slawinski (76) is for spheres. Packing data (75, 77) for spheres indicated at points *A* and *B* are: (*A*) Simple cubic, 6 points of contact per grain, $S = 0.5236$. (*B*) Rhombohedral, 12 points of contact per grain, $S = 0.7405$.

Also shown in Fig. 9 are the data of Crawford and Edward for filter paper (65). As with all the data represented in this figure, 0.1 *M* (or higher) KCl was used in the conductivity measurements. The actual nature of the filter paper with its ion exchange, swelling, and other properties (81) makes a complex conducting system even with the 0.1 *M* KCl electrolyte. If the fibers selectively exclude the ions when they swell and the paper is well rinsed with the buffer, then the resistance measurements are valid for calculating *T*. This is assumed by these authors. They computed R' for the volume v' of electrolyte which was added to a given weight and length of dry paper, using the middle member of equation (13) since the total volume of the wet paper cannot be conveniently measured. An empirical relationship between the equivalent of *S* and *T* was verified if about a 15% volume change for swelling is assumed. This empirical relation is shown over the range studied in Fig. 9. Under these conditions, their data indicate that the fibrous cellulose and glass filter paper can be obtained at lower *S* than can spheres in contact and that the structure coefficient is greater than would be expected for suspension of spheres at the same wetness.

At present, complete S and T data are not available for all the supporting media considered in this chapter. It is anticipated that with the starch and polyvinyl media, which have grains approaching the spherical shape, T will vary with S in a manner not too different from that for spheres. Approximately, for starch which swells ($W = 0.5$),

$$S = 0.7 \quad \text{and} \quad T = 0.5$$

(from Fig. 9); for nonswelling polyvinyl ($W = 0.4$),

$$S = 0.6 \quad \text{and} \quad T = 0.4$$

For gels, the support content is less than about 0.1. Since the curve for the asymmetric fibers of paper approaches that of the spheres at low S, it is expected that T for the gels without sieving will lie between $S/2$ and S.

G. Hydraulic Flow

If current flow can be used to determine the effective path length in a maze, then it would appear, at first, that hydrodynamic fluid flow could also be employed. However, there is a fundamental difference in the geometrical dependence of the two flow processes. The corresponding experimental relationship to Ohm's law is Darcy's law (75).

$$V \propto P \tag{19}$$

where V is the efflux volume per unit time for a given pressure difference P. As with Ohm's law it is convenient to take out the gross dimensions of the cell, and to use a high-conductivity fluid, so that there will be no electrokinetic effects. In this case the dependence on the nature of the fluid can be described by its macroscopic viscosity η, and so Darcy's law is written

$$v = \left(\frac{K}{\eta}\right) p \tag{20}$$

where

$$v \equiv V/q \tag{21}$$

is the "macroscopic efflux fluid velocity," and

$$p \equiv P/l \tag{22}$$

is the "applied pressure gradient" across the cell.

Here, as before, the area q and length l are macroscopic dimensions of the cell regardless of whether it is filled with a porous medium or not; K, defined by this equation, is the "specific permeability," usually called just the permeability. The ratio K/η and the functionally similar coefficient

K in Ohm's law can be contrasted for cells of various shapes of cross section containing free solution as well as for a cell containing porous media of various shapes of supporting particles. Let κ_0 and η_0 refer to the specific conductivity, and viscosity respectively, of the high-conductivity fluid in bulk. Consider first the free solution cells. Here, in current flow the size and shape of the cross section has no effect, and $\kappa = \kappa_0$. In fluid flow there is marked dependence on size, with K being proportional to the square of the radius r (from Poiseuille's law $\nu/p = r^2/8$). The dependence on shape is reduced if double the "hydraulic radius" m (82, 83) is used as a description of the cross section in place of r. Here m, the third geometrical parameter, is defined as the ratio of the volume of fluid in the cell to the wetted area of the cell (excluding the ends). For a cylinder,

$$m = \pi r^2 l / 2\pi r l = r/2 \tag{23}$$

For a porous conductor, the size or shape of the cross section has no influence on κ or K/η, but now the quantity and shape of the grains has a marked effect because of the channeling of the flow. The same factors, S and T, found sufficient for the description of the current flow given in equation (17), together with m are still not adequate for the fluid flow. Instead a fourth parameter, the "Kozeny constant" k_0 (83), must be introduced in order to write Darcy's law as

$$\frac{\nu}{p} = \frac{K}{\eta} = \left(\frac{m^2}{k_0 \eta_0}\right)\left(\frac{1 - S}{1 + T}\right) \tag{24}$$

Actually k_0 is not strictly a constant but rather a variable depending on the shape of the particles and their packing with a value between 3 and 8 (69, 73). Hence, the fluid flow cannot be used to evaluate T knowing S and m because k_0 is, in general, unknown. It should be noted, however, that fluid flow is used in the other way, assuming a k_0, to calculate first m from K, S, and T in equation (24) and then the area of the wetted surface from the definition of m. When written in a form suitable for this calculation, Darcy's law is usually called the Kozeny-Carman equation (83). With $S = T = 0$ and $k_0 = 2$, it reduces to Poiseuille's law for a circular pipe.

H. Electrokinetic Flow

Electrical current flow without fluid flow was considered under the operational definition of the structure coefficient T. Hydraulic fluid flow without current flow was just considered. A third type of process involves the cross relationship between electrical and fluid flow.

Mazur and Overbeek (84) and, independently, Lorenz (85, 86), have given the phenomenological description of such a combined flow process in general. The statements* are

$$\begin{aligned} i &= l_{11}e + l_{12}p \\ \nu &= l_{21}e + l_{22}p \end{aligned} \tag{25}$$

where the macroscopic potential and pressure gradients e and p are simultaneously acting to give a macroscopic efflux current density i and fluid velocity ν. Imagine an apparatus which will prevent the build-up of any potential difference across the region through which fluid is forced. Then (for $e = 0$) $l_{12} = i/p$ is the cross coefficient determined from "streaming current." Similarly, in an apparatus allowing no pressure head to develop ($p = 0$), $l_{21} = \nu/e$ is the other cross coefficient determined from "electroosmotic streaming." Mazur and Overbeek (84) show theoretically that $l_{12} = l_{21}$ from irreversible thermodynamics of interacting flows. Lorenz (85), on the other hand, derives their equivalence from the experimental Saxén's law

$$-\left(\frac{E}{P}\right)_{I=0} = \left(\frac{V}{I}\right)_{P=0} \tag{26}$$

He proposes to call the cross coefficient the "electropermeability" l_e:

$$l_e \equiv l_{12} = l_{21} \tag{27}$$

This equivalence has been verified experimentally for swelling and nonswelling porous media even though the regions of hydraulic and electrical transport do not completely overlap (84–88). From the description equation (25) and the relation equation (27) three other cross relationships like Saxén's law can also be derived, which may be useful in some calculations. For example, equation (26) can be used to calculate the pressure required to give the same fluid flow that a given voltage and current give by electroosmosis.† All experiments designed to measure either l_{12} or l_{21}, when performed (89) and interpreted (84) correctly are equivalent, for they yield the cross parameter l_e.

Bikerman, in his review (90), has given the derivation of Helmholtz, using electrostatic and hydrodynamic theory, for electroosmosis under no pressure head in a tube of large size compared with the (molecular) extent of the field produced by the immobile charges on the surface. The resulting relationship is:

$$V = \frac{\zeta\epsilon'}{4\pi\eta'}\left(\frac{q}{l}\right)E = \frac{\zeta\epsilon'}{4\pi\eta'}\left(\frac{v}{l^2}\right)E \tag{28}$$

---

* When given in terms of I, V, E, and P a capital is used for the coefficients. Then $L_i = l_i(v/l^2)$ where $i = 11, 12, 21, 22$.

† In this calculation, a convenient conversion factor is 1 watt/cm.^{3}/sec. = 9.869 atm.

Here ϵ' and η' are the dielectric constant and viscosity of the actual pore fluid and ζ is the zeta potential of the solid-liquid interface. The geometrical representation of the fluid space v/l^2 becomes for a porous medium the statistical v'/l'^2. Thus from the definitions of S and T, equation (28) can be written for a porous plug as:

$$\frac{V}{E} = \frac{\zeta\epsilon'}{4\pi\eta'}\left(\frac{v'}{l'^2}\right) = \frac{\zeta\epsilon'}{4\pi\eta'}\left(\frac{v}{l^2}\right)\left(\frac{1-S}{1+T}\right) \tag{29}$$

It is now possible to give the expression for l_e, since it is equal to $(\nu/e)_{P=0}$. First convert V and E of equation (28) to ν and e using equations (21) and (9). Then the electropermeability l_e is:

$$l_e = \frac{\zeta\epsilon'}{4\pi\eta'}\left(\frac{1-S}{1+T}\right) \tag{30}$$

Thus, again, a different measurement yielding l_e from an electrokinetic experiment does not enable evaluation of T from equation (30) since still a fifth parameter of the supporting medium, $\zeta\epsilon'/4\pi\eta'$ needs to be introduced.

Besides the cross coefficient l_e there is an additional effect of the ζ-potential. Neither the conductance l_{11} nor the permeability l_{22} are equal to the ordinary conductivity κ or permeability K/η of equations (17) and (24), respectively, measured in the absence of all electrokinetic effects. The difference is called, respectively, surface conductivity and electroviscosity. Lorenz (86) has given the phenomenological expressions for these terms and reviewed mechanistic formulations. These involve introduction of an additional factor beside the ζ-potential—namely, the thickness of the double layer. Thus, measurements of surface conductivity, e.g., yield new information, rather than simply the basic parameter T.

I. Absolute Determination of Zone Mobility

The preceding phenomenological or macroscopic description of the impregnated porous medium as a whole is necessary to emphasize the complex phenomena taking place because of the presence of the supporting medium. In the actual direct-current electrophoretic experiment, low ionic strength buffers are used, often in arrangements which permit the development of pressure heads. In such a case, calculations employing the current and/or the fluid flow must not neglect the sizable terms and complex coefficients in the complete description of the phenomena employed in equation (25). However, an understanding of the fluid and current flows can be used in indirect but important practical ways. In the first place a connecting tube of sufficiently large diameter between the electrode vessels will insure a negligible pressure head developing. Secondly, a constant current power supply will insure constant electrokinetic behavior, even though all of the current does not go through the migration path. In the event it is more practical in certain special cases to permit evaporation, then correction for the nonconstant fluid flow and conductivity can be attempted as outlined by de Wael (91).

The problem is now to consider the interior of the block a reasonable distance from the ends to avoid pH, conductivity, and volume changes possibly present (3, 10, 92). What is desired is to determine the "mobility of a solute zone." This is interpreted here as the "free-solution mobility" of that solute which would be observed in a straight channel in a solution of the same alternating-current conductivity and temperature as the fluid in the pores. It is important to control and measure the temperature, recognizing that mobility varies approximately as the viscosity, which is about 3%/°C. In the event the pores are extremely fine or that ion exchange takes place, then correction must be applied for the disparity between the composition of the buffer in bulk and the composition in the region of the pores available for migration.

The expression for calculating the mobility of a solute zone can be developed from the definition of "mobility" itself as velocity relative to the cell, if there were no net solvent velocity, per unit field strength and the concept of a statistical path length in the porous medium embodied in the structure coefficient T.

Imagine an ideal tracer of zero mobility to be in the initial solution zone placed at the position marked "Start" in Fig. 10. By whatever flow processes acting on the solution as a whole, let the zero mobility zone be moved a distance d_o measured with respect to the container. Similarly, denote the distance the solute zone moves from the start as d. Both of these quantities may be either positive or negative. Because of the structure of the supporting medium, the current path between these two zones

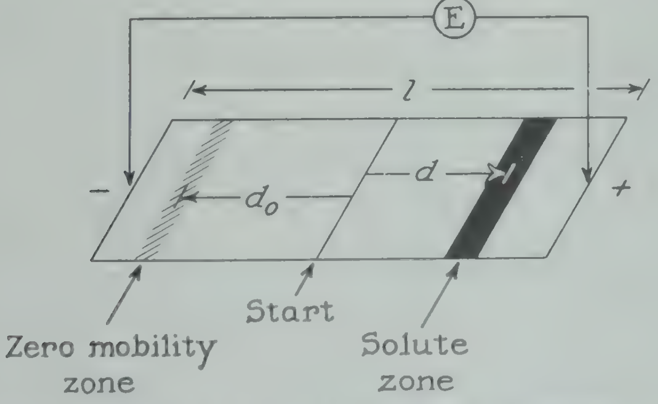

FIG. 10. Symbols used in the absolute determination of mobility.

is longer than $d - d_0$ by the factor l'/l and the potential gradient along this path is less than E/l by the factor l/l'. Using the notation as before, the average velocity in the average pore is

$$\left(\frac{d - d_0}{t}\right)\left(\frac{l'}{l}\right) = \left(\frac{d - d_0}{t}\right)\sqrt{1 + T} \qquad (31)$$

and the field strength in the pore is

$$\left(\frac{E}{l}\right)\left(\frac{l}{l'}\right) = \left(\frac{E}{l}\right)\left(\frac{1}{\sqrt{1 + T}}\right) \qquad (32)$$

Hence the mobility u in the average pore is

$$u = \left(\frac{d - d_0}{tE/l}\right)\left(\frac{l'}{l}\right)^2 = \frac{(d - d_0)l(1 + T)}{Et} \qquad (33)$$

Thus if T is known, the mobility can be calculated. It is well to state the assumptions involved in merely dividing the two expressions, equation (31) by equation (32):

(a) The velocity and field strength are constant. Usually the reasons for nonconstancy are evaporation and changes in voltage between the power supply and the portion of the block utilized for measurement. The details of methods to circumvent these technicalities are given elsewhere in this chapter.

(b) The conductivity of the solute zone is the same as that of the buffer, so the field strength is uniform. Complications arising from the requirement of constant regulating function have been considered by de Wael (91); [see also Longsworth (93)], and series and parallel combinations in the presence of electrokinetic effects by Lorenz (86).

(c) There are no volume changes along the block due to differences in ζ-potential in the various solute and buffer zones. It should be noted that local pressure differences can be set up, even though the over-all pressure head across the electrode vessels is zero (92).

(d) There is no molecular sieving or chromatographic retardation. It is not necessary to specify nonswelling, noninsulating media.

In general, it is not practical to convert equation (33) to a form employing the current, because the surface conductivity and other restrictions pose too severe conditions. It is useful, however, to apply equation (33 to zone electrophoresis employed as a relative rather than absolute instrument for the determination of mobility.

J. Relative Determination of Zone Mobility

Several colored proteins are available which can be used as known calibrating substances (94). Thus, for example, there are the various mobility types of hemoglobin and various derivatives of serum albumin.

either as its yellow form in certain fresh sera or dyed due to the binding of bromophenol blue. Colorless substances can also be used, provided that there is a specific assay or that the calibrating material is used in a different portion of the supporting medium. An especially useful technique is to use a mixture of two colored reference substances in a very small additional starting zone near the border of the block.

Let $\bar{u}$ be the known free-solution mobility of a reference substance not impeded by the supporting medium, by molecular sieving, or chromatography, and let $\bar{d}$ be the distance its zone moves from the starting zone. Equation (33) written for this case yields the structure coefficient T:

$$T = \frac{Et\bar{u}}{(\bar{d} - d_0)l} - 1 \tag{34}$$

This method was used to determine l' from T for sand by Winsauer *et al.* (95). The support content S cannot conveniently be determined in a relative manner and so is best measured directly by the displacement method, equation (4).

Dividing equation (33) for an unknown by equation (34) for the reference substance gives the expression for the mobility u of the unknown:

$$u = \left(\frac{d - d_0}{\bar{d} - d_0}\right) \bar{u} \tag{35}$$

With two reference substances the location d_0 of the zero mobility zone can be calculated from equation (35) [see references (10, 94)] irrespective of what flow processes cause its movement.

The macroscopic efflux fluid velocity ν of equation (21) can be calculated from the movement d_0 of the zero mobility zone, recognizing that $v = d_0q$:

$$\nu = \frac{v'}{qt} = \left(\frac{v'}{qt}\right)\left(\frac{d_0q}{v}\right) = \frac{d_0(1 - S)}{t} \tag{36}$$

If there is no pressure head between the electrode vessels then the migration of the zero mobility zone is due only to electroosmosis and equation (30) applies.

$$\frac{\zeta\epsilon'}{4\pi\eta'} = \left(\frac{1 + T}{1 - S}\right)\left(\frac{\nu}{e}\right)_{P=0} = \frac{d_0l(1 + T)}{Et} = u_0 \tag{37}$$

By comparison of equation (37) with equation (33), it can be seen that $\zeta\epsilon'/4\pi\eta'$ has the dimensions of mobility and hence can be called the "electroosmotic mobility" and be denoted by u_0.

Now consider the calibration of one medium knowing the parameters of another, assuming that the reference substance is not impeded by other than geometrical factors in the two media. The technique is to connect the two media in parallel to the same electrode vessels with no pressure head. Let a bar over a quantity refer to measurement in the known medium. Then from equation (33), written for both media, the unknown structure coefficient can be calculated as:

$$1 + T = \left(\frac{\bar{d} - \bar{d}_0}{d - d_0}\right)(1 + \bar{T}) \tag{38}$$

Similarly, from equation (37), the unknown electroosmotic mobility can be calculated from:

$$u_0 = \left(\frac{d_0}{\bar{d}_0}\right)\left(\frac{1 + T}{1 + \bar{T}}\right)\bar{u}_0 \tag{39}$$

If the same supporting material is compared at different wetnesses for which $u_0 = \bar{u}_0$ then both the solute zone and the zero mobility zone will move further in the wetter medium. This is an apparent paradox for the zero mobility zone, since, as one goes completely to free fluid, there is no net movement of the boundary marking a zero mobility (other than due to volume changes in the electrode vessels). This is because the moving boundary method averages out the electroosmotic and hydrodynamic flows through the cross section of the channel (96). There is no paradox when viewed in terms of the closed microscopic method (97) where the full electroosmosis is seen at the walls and the net flow across the channel is zero.

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Chapter 7

Preparative Electrophoresis without Supporting Media

M. BIER

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I. Introduction

There is little doubt that electrophoresis is at present the most efficient and elegant method for protein separation. On the analytical level, full advantage has been taken of its possibilities since the introduction of the Tiselius apparatus and the more recent development of zone electrophoresis. On the preparative level, progress has been much slower and it is only in the last ten years that this field has begun to receive widespread attention. This is borne out by the fact that Svensson (1) was able to present a thorough and detailed review of the literature on preparative electrophoresis up to 1948 in only about fifty pages. Even since then, the advance has continued to be relatively slow as compared to the analytical applications. For example, at present there are still merely a few proteins or enzymes that are prepared by electrophoresis in preference to other methods.* The amount of work that went into various salt and solvent fractionation schemes is out of all proportion to the effort put into the development of preparative electrophoretic methods, although a satisfactory electrophoretic technique would, in principle, be the ideal solution to all preparative problems.

The slow progress in the field of preparative electrophoresis is somewhat surprising since one of the most basic discoveries in this field considerably antedates the contributions of Tiselius. This is the observation of the phenomenon of electrodecantation by Pauli (2, 3). Yet, this method had not gained any wide popularity or attention from the protein chemists. In the subsequent twenty-five years, Pauli, Stamberger, and collaborators continued to study its application to various colloids, metal sols, etc., while at the same time perfecting its industrial utilization for the purification of rubber latices. Only with the work of Gutfreund (4), Cann and associates (5), Polson (6), and Bier (7), did this method and its various analogs arouse the protein chemists' interest.

Similarly, preparative electrophoresis on paper, powders and gels received momentum only after the description of the technique by Consden *et al.* (8), although in practice, if not in theory, it can be considered merely an extension of chromatography with the use of electric current.

Electrolysis and electrodeposition of metals and nonmetals, rather akin to electrophoresis in certain respects, are arts that are well mastered and practiced on the widest scale. What then are the reasons for the slow development of preparative electrophoresis? Basically, in all of electrophoresis there is only one problem, and that is the dissipation of the heat generated on the passage of the current. The heat has many undesirable consequences, most important being that unequal heating generates convection currents in the liquid which tend to mix the components already separated. Moreover, most enzymes and proteins are sensitive to heat. Both these factors are not operative in the case of either electrolysis or electrodeposition, and mixing is, in fact, favored, since interest centers in reactions occurring at the electrodes and not in the bulk of the solution.

The first successful solution to the dual problem of heat and convection was that of Tiselius (9). In his cell, the solution is stabilized against convection by a density gradient, the heavier protein solution being on the bottom of the cell. For efficient heat exchange with the surrounding cooling liquid, the cell is narrow and is operated at a temperature of about 4°C., at which the coefficient of expansion of saline solutions is close to a minimum. Nevertheless, there is a limit of about 0.15 watts of electrical power per cubic centimeter that can be dissipated in the cell. The Tiselius cell is, of course, usable not only for analytical, but also for preparative purposes. Numerous modifications of cell design were proposed, some of them capable of semicontinuous operation (10, 11), but the method has some inherent limitations, these being the above-mentioned maximum power tolerated and the fact that only the slowest and fastest components can be isolated, the recovery never being quantitative. Its unique advantage over all other methods of preparative electrophoresis is that the separation achieved can be optically registered prior to the isolation of the fractions. The method was partially reviewed by Svensson (1), and its technique is so similar to that employed in analytical work that it appears superfluous to discuss it in this chapter. Its most direct outgrowth in the preparative field is density gradient electrophoresis, which seems far more promising and will be considered later.

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* For instance, elastase and horse-radish peroxidase: Manual No. 9, Worthington Biochemical Corporation, Freehold, New Jersey.

A second approach to the control of convection currents is to be found in preparative electrophoresis on paper, powders, or gels. It is based upon the fact that very finely divided materials effectively block the gross flow of fluids and therefore also convection currents, while not affecting substantially the molecular kinetics, i.e. diffusion or electrophoretic migration. While the use of these anticonvection agents successfully solves the problem of convection, it does not in any way help in the problem of heat dissipation. Thus, there is a limit to the intensity of the electric field tolerated within each type of apparatus. This also sets a limit to the construction of large-size equipment because, with increasing cross section of the apparatus, and therefore increasing carrying capacity for fluid, the heat dissipation becomes more difficult and reduces the field intensity that can be applied. These problems are discussed in other chapters of this book. The advantage of these methods is that all the components of the protein mixture can be separated in a single run, if there is sufficient difference in the electrophoretic mobility of the components.

In the electrodecantation of Pauli (2, 3), neither of these two factors, i.e. temperature and convection, were controlled. On the contrary, the convection caused by the density gradient (due to the protein that concentrates close to the membrane, not due to temperature differences) is utilized for collecting the separated protein. The lack of any serious attempt to control the temperature in the many instruments designed has probably been one of the reasons for their unpopularity with protein chemists. It, however, did not hinder the preparation of purified rubber latex or inorganic colloids, which are less sensitive to heat and frequently have higher mobility. In electrophoresis-convection (5), the protein density gradient causes separation, and effective heat dissipation and control of undesired heat-convection currents were realized by confining the electrophoretic process to a relatively narrow channel. Similar results were achieved by Polson (6) through the introduction of cooling sections in the apparatus.

Electrodecantation as well as electrophoresis-convection permit the collection of essentially only two fractions: one containing all the mobile protein components, the other containing the isoelectric components. Thus they are merely concentration methods which concentrate the mobile components and withdraw them from the bulk of the solution. The purification of isoelectric components ensues, since these are not affected by the electric current and remain in the residual fluid. This limitation to two fractions only, an isoelectric and a mobile one, is a shortcoming in comparison to zone electrophoresis. However, since good control can be made of both temperature and convection factors, the principle can be effectively utilized for large-scale preparation of colloids and has already received industrial application in the field of rubber latices and tetrafluoroethylene polymers.

The latest newcomer in this field is continuous free-boundary flow electrophoresis (7). It has certain features in common with electrophoresis-convection, since both the temperature and convection elements can be successfully controlled. Its advantage is that it does not depend upon the density gradient of the protein film concentrated in the vicinity of the membrane in order to be operative. It depends upon the combination of a forced laminar flow of liquid perpendicularly superimposed upon the direction of the electrophoretic migration of the protein. As a result much higher rates of flow are obtainable.

The above methods do not, by any means, exhaust the entire field of preparative electrophoresis, various other principles having been proposed and utilized, as will be shown later. However, the review will not enter into those preparative electrophoretic procedures where a deposition of colloidally suspended material in solid form is achieved (12). These methods are frequently utilized for coating and insulation of wires, etc. Methods based primarily on electroosmosis will also not be considered. Some of these were industrially applied, particularly as a result of the efforts of Schwerin and the Gesellschaft für Elektroosmose, A. G. These early studies (from 1910) were reviewed by Prausnitz and Reitstötter (12a).

II. DENSITY GRADIENT ELECTROPHORESIS

The stability of the moving boundaries in the Tiselius cell depends upon the positive density gradients existing in the liquid column, due to the contribution of the dissolved proteins to the density of the liquid. The boundaries become unstable when the gradient across them becomes too small or even nonexistent. This is the main reason for the cell's limited usefulness for preparative purposes, as it is impossible to completely separate the components of a mixture into distinct zones. Indeed, every zone is characterized by a density gradient at each end, one being positive and the other negative. As a result, separation into zones always requires stabilization by other means.

At present, the favored means of stabilization in zone electrophoresis is by solid supporting media, such as paper, powders, or gels. The insoluble supports present certain difficulties, causing, for example, adsorption and electroosmotic flow, while the elution of the separated fractions is not always quantitative. Stabilization by a strong density gradient produced by an electrically neutral solute offers one means of obviating these difficulties. The gradient must be sufficiently strong to overcompensate for the negative gradients of the separated zones of the solute that is fractionated. One of the principal advantages is the ease with which a large number of fractions can be collected, the recovery being quantitative. Thus, it is well suited for both analytical and preparative purposes, even though, up to the present, it has not been widely employed.

The density gradient electrophoresis results in the formation of distinct zones, and can be carried out in some of the instruments suitable for electrophoresis with solid supporting media. Therefore, it could have been included in the preceding chapter, dealing with other forms of zone electrophoresis. However, of all the electrophoretic methods, this is the one most closely related to the original Tiselius method, i.e. its main distinction is that separation is attained in the absence of solid supporting media.

The use of stabilization by density gradients due to electrically neutral solutes was first advanced by Philpot (13). He explored the possibility of fractionating proteins in a system of continuously flowing superimposed layers of liquid, stabilized by density gradients, a strong electric field being applied perpendicularly to the moving liquid. The method never became functional, and its closest approximate is the forced-flow electrophoresis with a filter paper membrane which we shall discuss later.

Ericson and Nihlen (14) applied a concentration gradient of glucose for the stabilization of very dilute boundaries of cobalamins in the Tiselius cell. Sorof and Ott (15) also employed density gradients in the Tiselius cell. They found that a single sucrose boundary facilitated the isolation of the slowest-moving component in the descending arm of the Klett cell. Sorof *et al.* (16) then extended this work to a uniform sucrose gradient in the same apparatus. The advantage of this system is that it allows an optical visualization of the separated zones by the conventional schlieren optics. They tested the method with several synthetically prepared protein mixtures.

More suitable instruments were developed for this method. The simple U-tube arrangement utilized by Kolin (17, 18) is shown in Fig. 1. The bottom half of the tube is filled with a nearly saturated buffered sucrose solution, the top half with a sucrose-free buffer. A small volume of protein solution is layered between the two buffers in the position indicated by *M* in the diagram, by adjusting its concentration in sucrose to an intermediate value. The protein solution is not buffered, its conductivity therefore being small as compared to the conductivity of the under- and overlaying solutions. In this way, a much higher potential gradient is realized within this layer, and a very rapid separation of the colloids into zones of different mobilities is achieved. If the colloids are colored, a series of sharp bands will become visible within about 10 minutes. Kolin referred to these bands

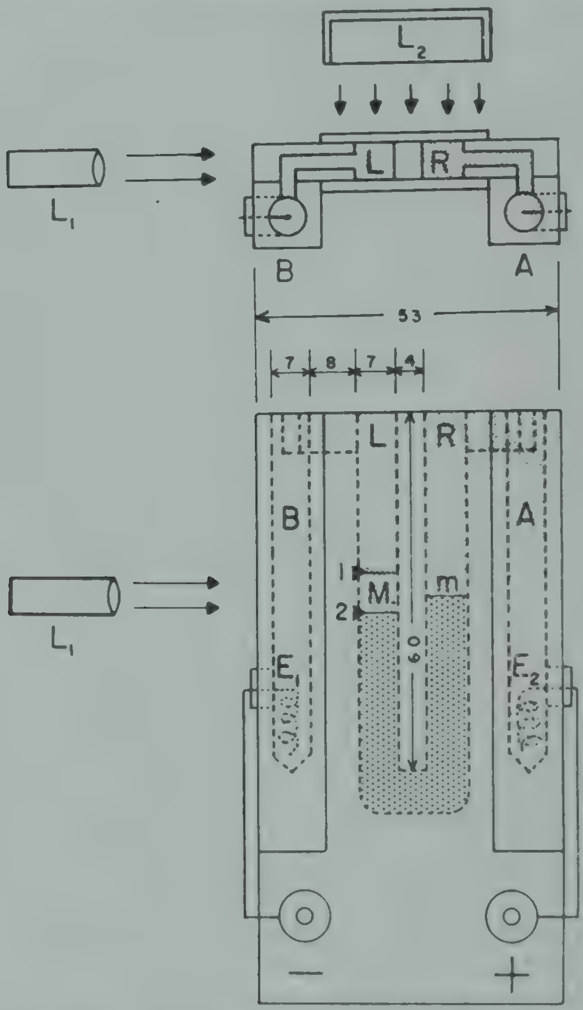

FIG. 1. Kolin's cell (18). L_1 and L_2, sources of light, E_1 and E_2; electrodes; *A* and *B*, channels filled with buffer; L and R, legs of the U-tube; *M*, mixture to be analyzed; *m*, boundary between light and heavy acid buffer; 1 and 2, marks indicating the positions of the boundaries of the mixture layer before separation. Upper section, top view; lower section, front view. The dimensions are in millimeters.

as "mobility spectra" (18, 19). After formation of the spectrum, it is possible to introduce a plunger into the tube in order to substantially reduce the cross section of the tube, thus causing the lines to spread over a greater length of the tube and facilitating their withdrawal with a micropipette (20).

By using, in the bottom of the cell, a sucrose solution buffered to a lower pH value than the overlaying solution, a pH gradient is superimposed to the density gradient (17, 18). In such a system if the current flows in the direction of increasing pH, the protein ions which are on the acid side of the isoelectric point move with the current toward the isoelectric point, being positively charged. The protein ions on the basic side of the isoelectric point are negatively charged and hence move contrary to the direction of the current, i.e. also toward the isoelectric point. Thus all protein ions within the pH gradient zone will converge toward the layer of liquid having the pH of their isoelectric point. In a mixture, a series of lines is obtained, referred to as the "isoelectric spectrum." Reversal of current polarity results in dilution of the protein. The isoelectric point of the solutes must, of course, be within the pH range of the gradient, or else mobility spectra result. A system of buffers has to be chosen, showing a minimum of migration of the pH gradient on application of the current. The isoelectric spectra develop within the region *M* of Fig. 1, while the mobility spectra develop outside this region. The formation of both types of "electrophoretic line spectra" was demonstrated with several dyes, colored proteins, as well as mixtures of algae and red blood cells (17–20), and Tuttle (21) employed this method for the visualization of human hemoglobins.

Brakke utilized the principle of density gradients in both centrifugal (22) and electrophoretic (23, 24) separations. For the latter, he designed simple U-tube cells, made of 18-mm. outside-diameter Pyrex glass tubing, and up to six tubes could be connected to the same electrode vessels. The density gradient was established by successively layering into both sides of the U-tube, solutions of decreasing sucrose concentrations, leaving the tubes overnight for the development of a smooth concentration gradient. A typical concentration gradient was about 50 gm. sucrose/l/cm., the over-all concentration ranging from 0 to 400 gm. per liter.

On the initial addition of the protein solution to such a column, a sharp zone is obtained, its very sharpness contributing to its instability. Because of the contribution of the protein to the density of its layer, this settles at a level where the sucrose concentration immediately above as well as below the protein zone is higher than in the zone itself. Sucrose diffuses more quickly into this zone than the protein diffuses out, producing gravitational instability resulting in the formation of droplets within the protein zone which fall to the bottom of the column. Brakke (23) found that the best way to overcome this difficulty is gently to stir the protein layer whenever the formation of droplets becomes visually apparent, in order to broaden the zone. After about an hour, stirring becomes unnecessary because a smooth sucrose gradient has been re-established. This method was utilized for the analysis of plant viruses, and it was shown that in buffered solutions the migration rate is independent of sucrose concentration. The resolution of the method was tested with human serum, and, while the results were good, they did not compare with those obtainable by other methods.

Svensson and Valmet (25) applied the same principle for preparative purposes in the Svensson column electrophoresis apparatus.* An excellent separation of four anionic dyes was obtained in a water-ethanol system. For the formation of the density gradient, Svensson and Valmet employed a mechanical device of the type proposed by Bock and Ling (26) for gradient elution chromatography. In a later paper, Svensson *et al.* (27) discussed extensively the stability conditions in density gradients and applied the method to the fractionation of serum proteins. A maximum of 7 ml. of protein was used in single runs containing up to 250 mg. of protein. For proper separation, 23 hours at 410 volts, 30–15 milliamperes, were required.

Excellent separation of serum proteins was obtained by Berg and Beeler (28). They also employed a commercially available column electrophoresis apparatus† and utilized a very simple arrangement for the formation of the variable density gradient. The columns could be utilized immediately after filling, without the overnight equilibration. Comparative patterns of density gradient and Tiselius electrophoresis are reproduced in Fig. 2. A maximum of 1 ml. of sample containing 50–300 mg. of protein was fractionated, the time required being 18 hours. The patterns presented best illustrate the potentialities of the methods. The hope is that it will be possible to design suitable instruments to permit the fractionation of large volumes.

III. ELECTRODECANTATION

A. Principles and Methods

The phenomenon of electrodecantation was discovered by Pauli in the course of his pioneering investigations on the purification of colloids by electrodialysis. Utilizing the cell depicted in Fig. 3, he observed that when a resting colloid solution is subjected to a passage of direct current, stratification takes place, the solution separating after a while into two distinct

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* LKB-Produkter Fabriksaktiebolag, Stockholm 12, Sweden.

† Macalaster-Bicknell Co., Cambridge, Massachusetts.

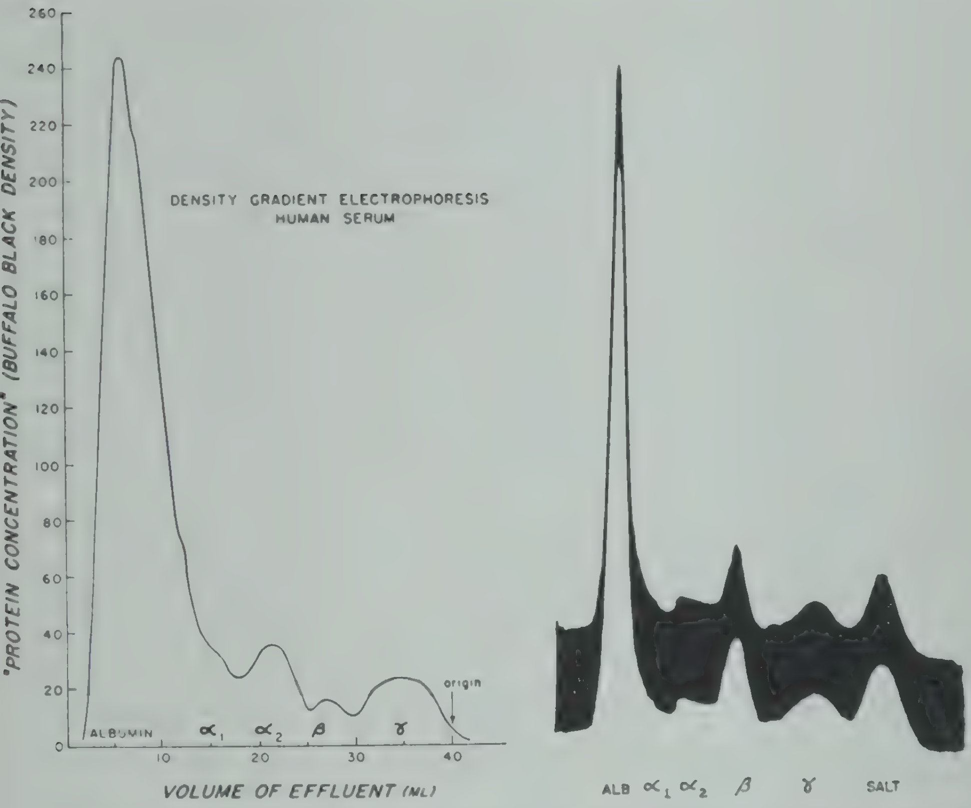

FIG. 2. Electrophoresis in density gradient of human serum compared with free electrophoresis (28). 0.05 *M* tris buffer at pH 8.6 at 200 volts and 4 ma. for 18 hours. Tiselius electrophoresis performed in diethyl barbiturate buffer with Γ/2 = 0.1 for 55 minutes.

layers, the bottom one containing most of the colloidal matter (2). The two layers could be distinguished either by the color of the colloid (hemoglobin) or by the gradient of the index of refraction, which was sufficiently sharp to be visually observable. Adolf and Pauli (3) first reported this finding for serum albumin, but most of the subsequent work from Pauli's Institute for Medical Colloid Chemistry of the University of Vienna dealt with the simultaneous purification by electrodialysis and concentration by

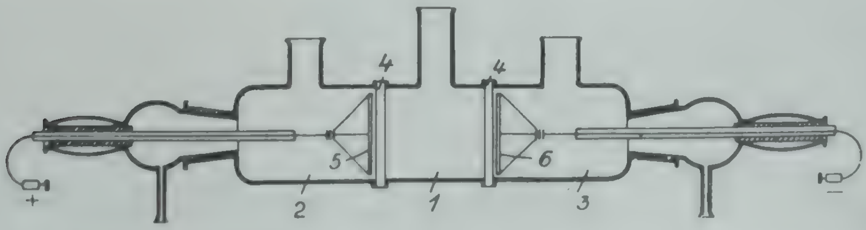

FIG. 3. Electrodecantation cell of Pauli (2, 35). *1*, Electrodecantation compartment; *4*, semipermeable membranes; *5* and *6*, electrodes.

electrodecantation of various inorganic sols, such as gold, silica, sulfur, etc. Some of the achievements were summarized by Pauli (29).

Samec and co-workers (30, 31) applied Pauli's discovery to the fractionation of starch. By submitting a 2% potato starch solution to electrodialysis, with an electric field of 36 volts per centimeter, a viscous, gel-like bottom layer was obtained which contained the amylopectin fraction, while the fluid top layer contained the amylose. The separation is due to the electric charge of the amylopectin fraction, which is the only one that contains organic phosphorus. They employed this method to determine the two fractions in a number of different starches. Dahl (32), in an apparatus of his own design, later confirmed these results and found that the separated amylose contained only 0.001%, while amylopectin contained 0.067%, of bound organic phosphorus.

The phenomenological basis of electrodecantation was explained by Blank and Valko (33). Under the influence of the electric field, the colloidal particles migrate toward one of the semipermeable membranes separating the center compartment from the electrodes. As a result, in the immediate vicinity of this membrane a layer of increased colloid concentration is formed which, because of its greater density, sinks to the bottom of the compartment. At the same time, close to the opposite membrane a layer devoid of colloids will have formed, which will rise to the top of the compartment. The concentrated layer at the bottom of the vessel, and the colloid-poor layer on top are stabilized by the density gradients, and no substantial mixing takes place. This explanation was soon confirmed by Verwey and Kruyt with the help of an ingeniously constructed apparatus (34). The problem of heat generated in the center compartment was partially avoided by the use of inorganic colloids which are less sensitive to it. Moreover, the experiments were carried out with unbuffered solutions, electrodialysis occurring simultaneously with electrodecantation. Therefore, only small current densities were operative, although relatively high voltages were applied (36 volts per centimeter) (30). As a result, total power consumption and heat generated were small.

Although the batch capacity of the instruments was high, still higher production rates were attained with a semicontinuous apparatus, described in 1939 (29, 35). The apparatus consisted of three sections, the process of electrodecantation taking place only in the center section. The colloid solution was fed continuously into this center section close to one of the membranes, and the concentrated colloid was withdrawn periodically from the bottom compartment. The colloid-impoverished portion of fluid was allowed to overflow continuously from the top compartment. With this apparatus, the rather large amount of 4–5 liters of goldsol could be concentrated to only 100 ml. in about 10 hours (36).

All of these experiments were carried out with colloids of a specific density greater than water, which collect on the bottom of the electrodecantation vessel. Applying the same method to rubber latex, Pauli and Stamberger (29, 37) observed that it collects on the top of the vessel in accordance with its specific density of less than 1. Advantage was taken of this fact in industrial applications, which will be reviewed in the next section. The striking implication of this method is that it permits a separation according to the specific gravity of the colloids, proteins in the latex sinking to the bottom (37). Since the process of electrodecantation occurs only at the membranes, it was apparent that a considerable increase in the capacity of the instruments could be obtained by multiplying the number of membranes. Several such instruments were designed by Stamberger and Schmidt (38), Ansell and collaborators (39), and others, and they will be discussed in the following section. A laboratory multicell apparatus was recently described by Stamberger (40), which utilizes the commercially available Webcell electrodialyzer.*

Another way in which electrodecantation may be used for separating various colloids was shown by Gutfreund (4), who pointed out that any isoelectric proteins would not be affected by the passage of electric current. Utilizing buffered mixtures of ovalbumin and hemoglobin at the isoelectric point of the latter (pH 6.8), he obtained a partial separation of the two proteins in an apparatus essentially similar to the original Pauli electrodialysis apparatus depicted in Fig. 3.

A considerably more sophisticated electrodecantation apparatus was described by Polson in 1953 (41, 42). In it, he has maintained the multimembrane principle of Stamberger, spacing the cellophane membranes about 1 mm. apart with the use of plastic frames. Each block of these membranes, containing about eighteen to twenty such frames and membranes, is separated from the next block by a spacer through which cold buffer is circulated. In this way, Polson obtained not only a certain amount of temperature control but also an improved control of the pH and buffer composition. Such a multimembrane apparatus is reproduced in Fig. 4. Typical dimensions of the apparatus are: membrane spacings, 0.75 mm.; height of the cell, 45 cm.; width, 9 cm.; and thickness of the multimembrane compartments, 2.5 cm. (43). Water-tightness is assured by placing rubber gaskets between the different compartments. In order to reduce thermal convection within the apparatus, the electrodes are not parallel but are closer at the top than at the bottom. In this arrangement, there is a greater flow of current through the top of the apparatus than through the bottom, and therefore more heat is generated at the top. The temperature gradient, in addition to the concentration gradient of the protein itself, is claimed to reduce undesired convection currents. Moreover, it was found that greater efficiency of separation was attained with the apparatus working in an oblique position. It is explained that in this way the layer of increased colloid concentration formed at each lower membrane has a reduced tendency to "fall back into solution."

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* Brosites Machine Co., New York, New York.

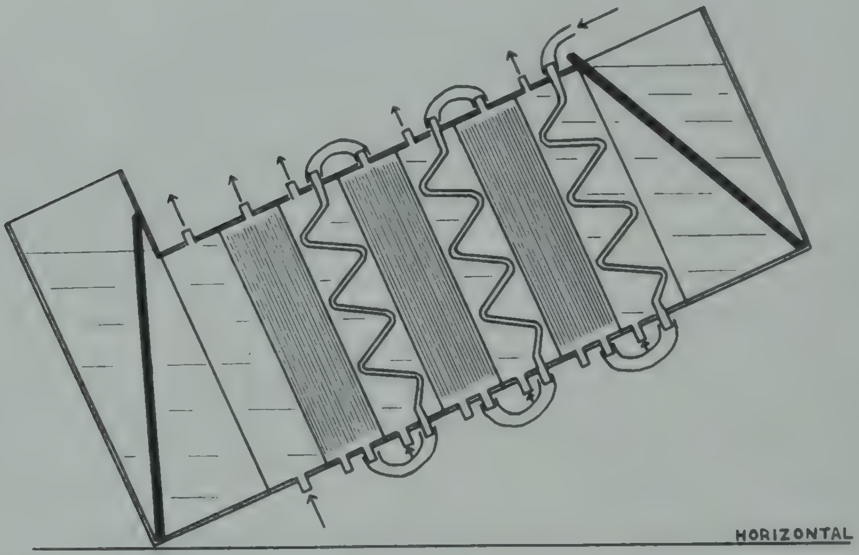

FIG. 4. Multimembrane electrodecantation apparatus of Polson (6, 42). The supernatant from the first separation section is passed into the bottom of the second, and the supernatant of this section is passed into the bottom of the third. The supernatant from this section passes into a receiver.

In each multimembrane section, all the subcompartments formed by individual membranes intercommunicate at the top and bottom, resulting in simultaneous withdrawal of the contents. In an apparently favored arrangement, there are three sections of about twenty membrane units each, the protein being continuously fed through all three, connected in series. The apparatus can also be utilized in batchwise operation. It is claimed that the quantitative recovery of the isoelectric protein is partially favored by a displacement phenomenon, i.e. the apparent displacement of the isoelectric protein from the bottom layer by the accumulated mobile protein. Thus on batch-electrodecantation of a mixture of ovalbumin and hemoglobin at the isoelectric point of the latter, only traces of hemoglobin were apparently found to contaminate the separated concentrated ovalbumin.* It was also observed that not only proteins but also electrolytes accumulate at the bottom of the apparatus. This is due to the charge of the membrane, which hinders the unrestricted passage of negatively charged ionic species. As a result, in the vicinity of the membranes facing the anode, the anions will accumulate in greater concentration than in the bulk of the liquid and will also decant to the bottom. This effect can be partially prevented by using buffers containing divalent cations, such as magnesium, which decrease the zeta potential of the membrane. The applications of this method to protein fractionation will be reviewed in Section III, C.

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* This phenomenon was not observed in electrophoresis-convection.

B. Industrial Applications of Electrodecantation

Electrodecantation received its first industrial applications in the field of rubber latex technology, due to the combined efforts of Pauli, Stamberger, and collaborators, as sponsored first by "Semperit" Österreichisch-Amerikanische Gummiwerke, A. G. (44) in Vienna, and later by Dunlop Rubber Co., Ltd., Birmingham, England (45). This application was partially anticipated by McGavack who in 1933, received a patent (46) for the concentration and purification of latex by electrical means. The latex was exposed to either direct or alternating current in a simple square box, the electrodes being immersed directly into the latex. A steady flow of latex was maintained through the box, and it was claimed that the treated latex creamed considerably faster than the controls. From the description given, it is hard to judge whether any electrodecantation was effective in the process, or whether the results were due to other changes produced by the electric current.

In the electrodecantation of rubber latex, advantage is taken of the low specific density of the rubber particles, which rise along the semipermeable membranes and can be collected as a cream. Initial results were obtained with the use of the simple Pauli electrodialysis apparatus (Fig. 3). It was observed that the efficiency of the layering depends, to a large degree, upon the concentration of latex and the electrical potential applied. At high rubber concentrations and high potential, no stratification was observed (latex density 60% solids, 5 volts per centimeter), but all of the rubber was found deposited on the membranes. At low concentration and density (5%, 0.3 volts per centimeter), complete stratification was obtained. Intermediate values of density and potential gave a partial stratification with varying degrees of rubber loss due to deposition on the membranes. With a periodic reversal of the polarity of the electric field, higher potentials were tolerated, as reversibly precipitated rubber is brought back into solution (47).

Considerable work was done by Pauli, Stamberger, and their associates on the development of high capacity equipment. The first steps in this direction were the introduction of large numbers of membranes into the path of electric migration (38), periodic reversal of current polarity (37),

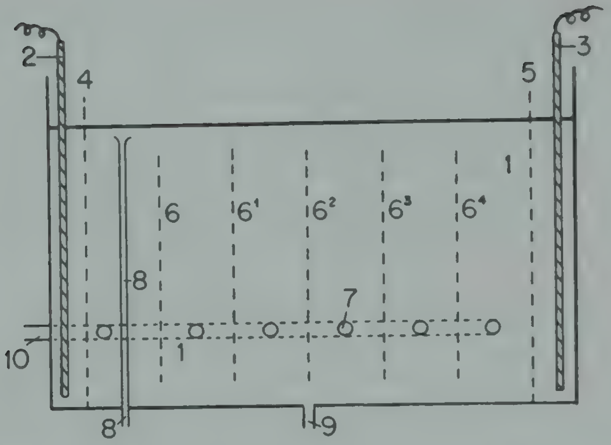

FIG. 5. Multimembrane electrodecantation apparatus of Stamberger and Schmidt (38).

and arrangement for the continuous inflow of the latex, and continuous withdrawal of the separated cream (36). A number of different ways of arranging membranes were proposed. A typical arrangement is illustrated in Fig. 5, and the finished apparatus in Fig. 6. The membranes are suspended into the apparatus from the top and can be easily replaced.

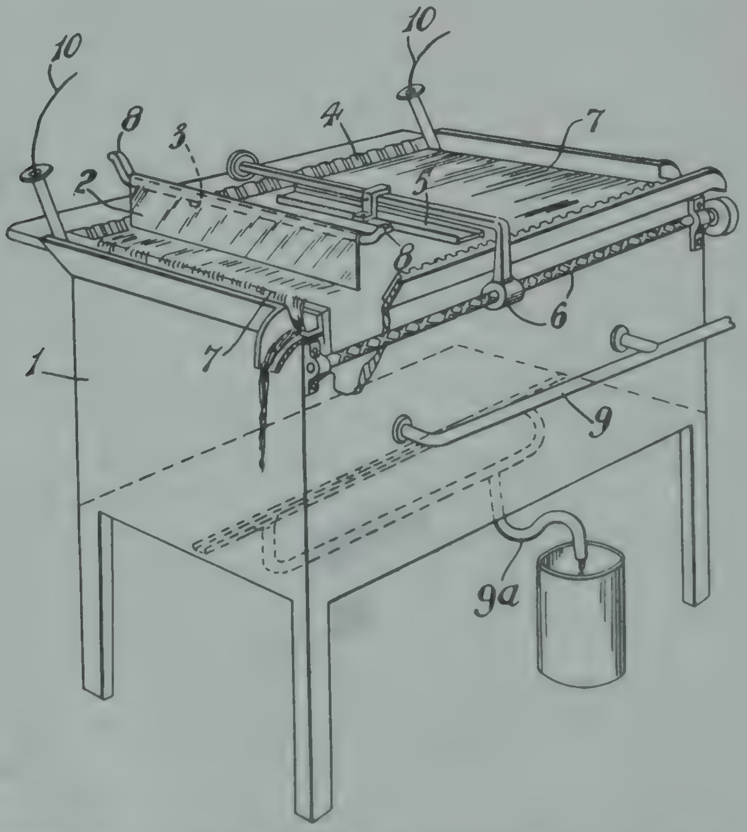

FIG. 6. Electrodecantation apparatus for latex according to Ansell *et al.* (39)

Typical results obtainable with such an apparatus were given in a review by Murphy (47). The runs were carried out with a latex of about 30% dry rubber content in 0.5% ammonia solution, in an apparatus having an hourly output of about 2 gallons. The data are reproduced in Table I.

TABLE I

ELECTRODECANTATION OF LATEX[a,b]

Hours run	Output per hour (kg.)			Total solids (%)		Current strength	
	Cream	Serum	Total	Cream	Serum	Volt	Ampere
1	9.0	10.0	19.0	52.6	11.0	3.0	7.0
4	9.0	10.0	19.0	59.2	8.3	3.0	8.8
10	8.9	10.0	18.9	61.8	7.3	3.1	10.5
50	10.4	10.0	20.4	62.0	5.1	3.0	13.0
100	9.2	10.0	19.2	60.1	6.6	3.0	12.5

[a] According to Murphy (47).

[b] Average dry rubber content of latex (0.5% NH_3) = 30%
Average dry rubber content of cream = 59.7%
Average dry rubber content of serum = 1.5%

Larger units were constructed with an output of 7 gallons and, in practice, several such units can be fed simultaneously from a central latex supply and have a current reversal system. Similar results can be achieved by centrifugation. Electrodecantation was claimed by Murphy to be superior, since, under production conditions, the loss in serum rarely exceeds 1%, while in centrifugation the loss in the skim may be as high as 7%. This is particularly important for the preparation of highly purified latices of low electrical conductance, where it may be necessary to dilute and recream the concentrate more than once. The degree of separation also compared favorably with that obtained by the action of creaming agents on the latex, with the advantage that the cream is obtained free of these contaminants.

It was claimed that in the process of electrodecantation of rubber latex, a separation of rubber and the accompanying proteins is obtained, the latter sedimenting to the bottom. Nevertheless, the properties of the latices obtained by centrifugation and by electrodecantation are remarkably similar, as can be seen from the data presented in Table II (47), and in commercial use they are interchangeable.

In a later review, Murphy (48) reported that commercial production units were operated by Dunlop at two rubber plantations in Malaya. Impressive photographs of the installation were presented, as shown in

TABLE II

Properties of Latex Concentrates[a]

	Once centrifuged latex	Once electro-creamed latex	Twice centrifuged latex	Twice electro-creamed latex
Alkalinity (% NH_3)	0.63	0.59	0.67	0.66
Total solids (%)	61.3	61.4	61.7	61.8
Dry rubber content (%)	59.7	59.8	61.5	61.5
Ash (%)	0.45	0.40	0.14	0.14
Nitrogen (%)	—	—	0.22	0.23
Color of latex	Grade 1	Grade 1	Grade 1	Grade 1
Color of film (Lovibond units):				
Unaged	1.5	1.0	1.0	< 1.0
Aged 16 hours at 95°C.	2.5	2.5	1.25	1.5
Z. O. T.[b] stability	64	63	14	15

[a] According to Murphy (47).

[b] Zinc oxide test.

Fig. 7. They could be operated continuously for over 170 hours before cleaning, with an over-all separating efficiency of 97% dry rubber content. The temperature rise in solution during electrodecantation was not sufficiently high to necessitate cooling the entire apparatus. Precooling of the latex was sometimes helpful. No total production quantities were given. The similarity of electrodecanted and centrifuged latex was again emphasized, though electrodecantation resulted in significantly higher mechanical stability. Electrodecantation is again claimed to produce smaller losses of rubber in the skim, and the average particle size of the decanted cream is smaller.

The charge of the latex particles is, of course, not due to the rubber itself, but to the other solutes. Thus, increasing amounts of ammonia normally added to the latex as a preservative, raise the mobility of the particles. Ionic surface-active agents have the same effect and are frequently used. Not only can they increase the charge of the particles, but they can even reverse their sign. Particles of synthetic rubber frequently have very low charge resulting in poor separation on electrodecantation, but this can be improved by the addition of suitable detergents to the solution. The use of detergents is claimed to have the added advantage of reducing the tendency to deposition of the latex particles on the membranes (47).

Electrodecantation has found further industrial use in the concentration of aqueous dispersions of polytetrafluoroethylene (49). A typical solution

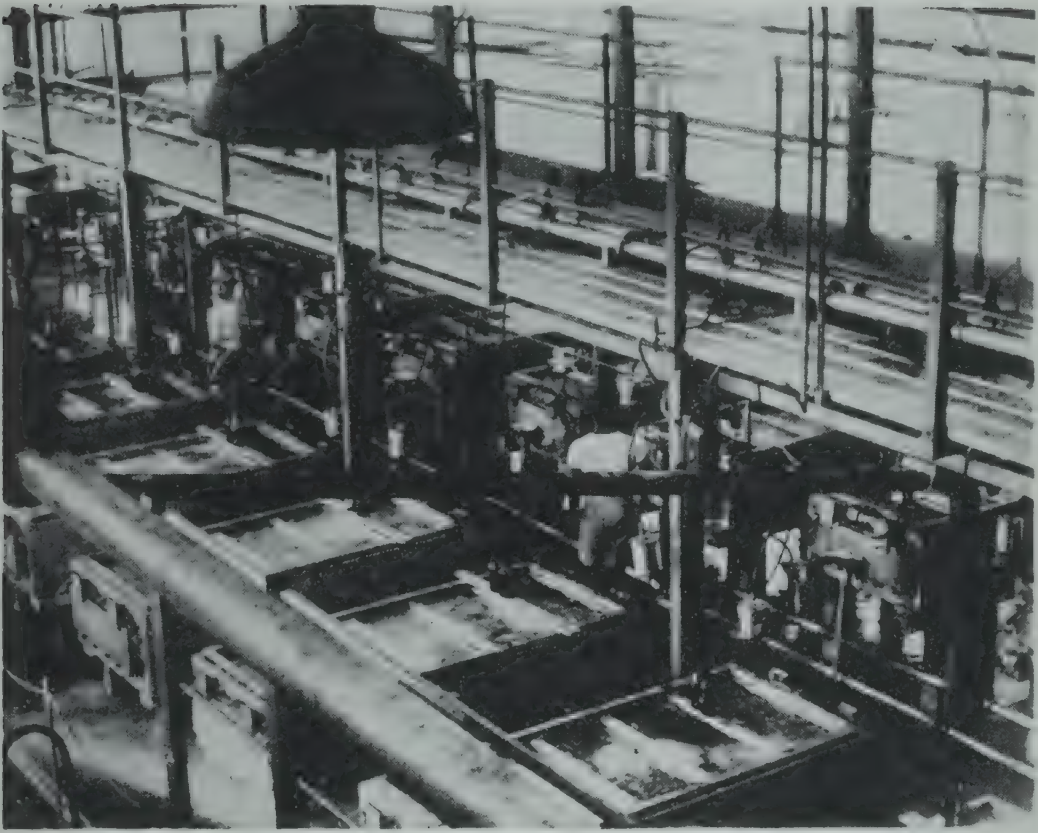

FIG. 7. Electrodecantation plant for latex creaming in Malaya. General view of electrodecantation units. From Murphy (48).

contains about 4% by weight of the polymer, and 0.2% of sulfonic detergent, primarily sodium laurosulfonate. After electrodecantation, the concentrated suspensoid (owing to its higher density, it decants to the bottom of the apparatus) contains from 55% to 74% polytetrafluoroethylene, and in order to prevent deposition at the membranes, periodic reversal of the polarity of the current is practiced. The maximal concentration that can be achieved in the apparatus is limited to about 74% dry weight, since the colloid coagulates at higher concentration. The patented apparatus differs little from those previously described except for the provision of collecting the concentrated suspensoid at the bottom rather than at the top of the apparatus. Also added were means to recycle part of the bottom concentrate by adding it to the dilute original suspension, thus achieving a higher final concentration. A semiproduction cell is described, having stainless steel electrodes 76 cm. apart and a plurality of intermediate membranes with a total effective area of about 13840 cm.^{2} The voltage applied was 150 volts, giving a potential gradient of 1.97 volts per centimeter. The direction of the current was reversed every 3 minutes.

The rate of flow input was 9.88 liters per hour, resulting in a 94.8% yield of dispersed polytetrafluoroethylene in concentrated form. Again no special provisions were made for temperature control during the process of electrodecantation. A production unit (50) consists of a tank in the shape of a rectangular prism about 60 cm. long, 12 cm. wide, and 24 cm. deep. On the bottom of this is fastened a pyramid-shaped section which tapers in both dimensions to a 1.2 cm. opening and serves for the collection of the concentrated polymer. The tank has 170 membranes, and a potential of 250 volts is applied. The starting material is a 35% suspensoid and it is concentrated to 60% with a very high (over 95%) average efficiency. The yield of one of these units is about 500 pounds of 60% suspensoid per 8-hour shift. No annual production figures are available.

C. Applications to Proteins

The only significant applications of electrodecantation to proteins were reported by Polson and his collaborators. In a batch-operated vertical separation cell, human sera were electrodecanted at pH 6.6, the mean isoelectric point of γ-globulin in a phosphate-$MgSO_4$ buffer, with an average voltage gradient of 5 volts per centimeter across the membranes. At the end, the content of the apparatus was separated into a bottom layer of about 100 ml. and a top layer of about 400 ml. After about 3–4 hours of operation, there appeared to be a 100% yield of γ-globulin in the top layer, contaminated by about 30% of the other protein components of the serum. After 7–9 hours of operation, the yield of γ-globulin decreased to 60–70%, with only about 5% of contamination. After 17 hours, the γ-globulin yield was only about 30%, but no electrophoretically detectable impurities were found. The decreased yield of γ-globulin was probably due to the progressive lowering of the pH of the buffer during the electrodecantation process. In another series of experiments, by the combination of sodium sulfate precipitation and electrodecantation, various electrophoretically homogeneous components of serum were obtained, notably serum albumin, γ- and α-globulin by repeated electrodecantation at pH values of 5.4, 6.6 and 8.0, respectively. In Fig. 8 are presented the diagrams of initial serum and the γ-globulin prepared in a continuous flow apparatus, with a flow rate of 0.45 liter per hour and 0.22 liter per hour, respectively, at pH 8.6. The sample obtained at the higher rate of flow was still heavily contaminated with the other protein components of serum, while a pure component was obtained at the slower rate of flow. The yield of the γ-globulin fraction was not indicated (41).

The application to viruses presented two difficulties. For one, at excessive field strength the virus seemed to attach itself irreversibly to the membranes toward which it moved. Also, the concentration of the virus

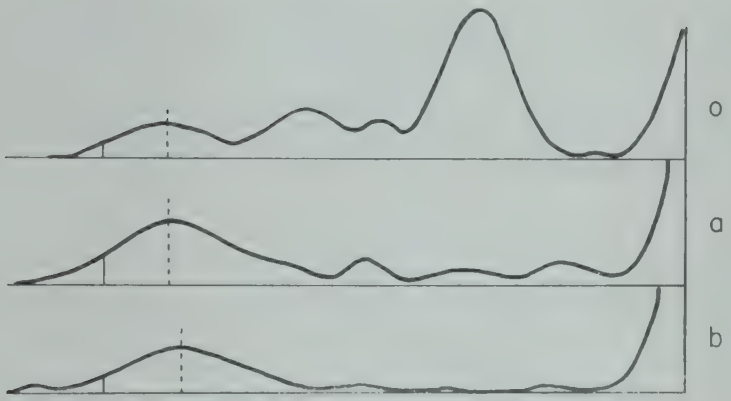

FIG. 8. Electrophoretic diagrams of γ-globulin prepared by electrodecantation (6). *o* = original serum; *a* = γ-globulin separated at flow rate of 0.45 liter per hour; *b* = γ-globulin separated at flow rate of 0.22 liter per hour.

being too small to give a concentration gradient and sediment to the bottom, it was necessary to add a carrier protein moving in the same direction as the virus, and the latter was then concentrated at the bottom, swept by the added protein. In practice, the operation was found to be suitable only as a preliminary concentration step in the purification of MEF-1 poliomyelitis virus (41).

In an application to enzymes, trypsin was chosen as the object for isolation (41), being characterized by a high isoelectric point (51). Bovine frozen pancreas was extracted in a sodium glycinate buffer, pH 10.8, with 0.025 *M* calcium acetate, and the centrifugate purified by electrodecantation in a batch apparatus. After 5 hours, the supernatant was separated, dialyzed against concentrated $(NH_4)SO_4$ and $MgSO_4$ solution, trypsin crystallizing out in the dialysis bag. Thus a single-step isolation of trypsin was achieved, although about 75% of activity was lost in the process.

In another paper, Polson (43) reported the purification of deoxyribonuclease, its isoelectric point having been determined to be at about pH 4.7. A crude extract of the pancreas was utilized for the purification. The procedure employed was as follows: the entire apparatus was first filled with 20 liters of 0.025 *M* acetate buffer, and an average potential gradient of 5 volts per centimeter was established across the electrodes. After temperature equilibrium was established in the cell (nonparallel electrodes having been used), the crude extract was introduced into the bottom of the apparatus at the rate of 300 ml. per hour. When the protein first appeared in the top effluent, the inflow of enzyme solution was replaced by an inflow of a 10% glucose in the acetate buffer. This flow of glucose was maintained until about 80% of the cell content was displaced by glucose. The effluent collected was brought to 70% saturation with ammonium sulfate and the precipitate collected. It was dialyzed in distilled water, the yield of the enzyme so obtained having been 0.7 gm. per 20 lb. of pancreas. No reasons were given by the author for the selection of this cumbersome procedure, a mixture of batch and continuous operation. The final product was found to be homogeneous on electrophoretic and ultracentrifugal analysis.

In the same laboratory, Largier (52) has purified tetanus toxin from crude concentrated filtrates of *Clostridium tetani* at the isoelectric point of the toxin (acetate buffer, pH 5.1, $\Gamma/2 = 0.05$). The flow through a single multimembrane separation cell was maintained at 60 ml. per hour with a potential gradient of about 3 volts per centimeter. In a second step, the toxin was concentrated under similar flow conditions, but using a phosphate buffer (pH 7, $\Gamma/2 = 0.07$) in the presence of magnesium ions. After a final ammonium sulfate precipitation, the toxin obtained was apparently homogeneous on electrophoretic and ultracentrifugal analysis, as well as by gel diffusion tests. The method of electrodecantation was applied also to the purification of diphtheria toxoid (53) and tetanus antitoxin (54).

IV. Electrophoresis-Convection

A. Principles and Methods

Of all the methods discussed in this chapter, electrophoresis-convection received the widest application in the field of proteins. This was largely due to the efforts of Kirkwood and his collaborators. It was, indeed, Kirkwood who first proposed a new method of protein separation, named electrophoresis-convection, based on a combination of electrophoresis and thermal diffusion (55). The protein, placed in a long and narrow channel, was supposed to be subjected to horizontal transport by electrophoresis, and to vertical transport by thermal gradient, outwardly imposed through the two side walls of the channel. In the initial experimental tests of the theory, it was observed that the temperature gradient was unnecessary, since the density gradient produced by concentrating the protein at the membrane was far greater than that produced by the temperature differential (56). A circular electrophoresis-convection apparatus was utilized, the cell consisting of two concentric Visking cellulose tubes of 19 and 24 mm. diameter, and about 16.5 cm. long. With this apparatus, substantial concentration of horse hemoglobin, bovine serum albumin, and an azo-ovalbumin was achieved, as well as partial separation of a hemoglobin-serum albumin mixture. It is interesting to note that the method originated as an attempt to apply to proteins the principles of separation of isotopes by thermal diffusion employed in the Clusius column, rather than through a further development of the electrodecantation experiments of Pauli.

The apparatus employed in most of the later work was that described by Cann *et al.* (5) in 1949, and its cell is presented in Fig. 9. The cell consists of an upper and lower compartment connected by a vertical narrow channel formed by two parallel semipermeable membranes, held in place by two face-plate assemblies. The channel communicates with the compartments through narrow slits. The frame of the apparatus is made of Lucite, and nylon screws are used for assembling the face plates.* The capacities of the upper and lower compartments are 100 and 50 ml., respectively, the height of the channel is 24 cm., and the width 5.1 cm., the distance between the membranes being 0.1–0.2 cm., depending on the

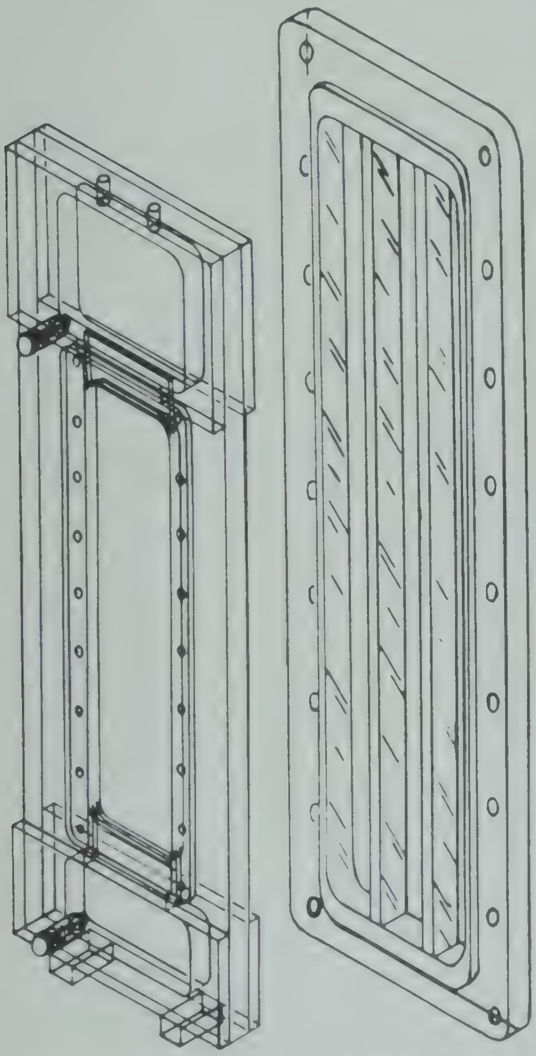

FIG. 9. Electrophoresis-convection cell of Cann *et al.* (5). Left: cell block assembly. Right: face plate used for securing membranes to the cell.

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* Beckman Instrument Co., Pasadena, California.

hydrostatic pressure. While these dimensions could be varied, there are few experimental data on the possible effects (57). The entire cell is immersed in a refrigerated circulating buffer, and an electric field is applied across it by means of two external platinum electrodes coextensive with the membranes.

In operation, the apparatus is filled with the protein solution to be fractionated, the upper compartment not being necessarily completely filled. Circulation of buffer and the electric field are then established. On conclusion of the experiment, first the top compartment is emptied with a syringe and then the bottom compartment is drained by means of an appropriate valve.

Fractionation of a heterogeneous mixture of proteins can be effectuated in two ways. By the isoelectric procedure, conditions of pH and buffer composition have to be so chosen that the protein, which is desired in a high state of purity, be at its isoelectric point, all other proteins moving either as cations or as anions. Under the influence of the electric field, all the mobile components will migrate toward one of the membranes, form a layer of increased concentration in the immediate vicinity of this membrane, and sink into the bottom compartment by gravity-controlled convection. An equal volume of protein solution will thereby be displaced from the bottom compartment and rise into the channel, where it will be exposed to the electric field. Through this principle, a continuous convective flow is established involving both compartments. At the end of the experiment, all mobile components will have accumulated in the bottom compartment, while the isoelectric protein will not have been affected by the electric field. It will, therefore, be the only component still present in the upper compartment and can be withdrawn from it in a high state of purity. If desired, this top compartment can be again purified in a second stage. While this procedure is by far the most efficient, it suffers from the disadvantage that only the two terminal protein components (in terms of the distribution of isoelectric points on the pH scale) can be obtained in a pure state. The components with intermediate electric points in a multicomponent mixture cannot be isolated by this procedure directly since accumulation of proteins simultaneously at both membranes disturbs the convective flow. Fractionations are mostly carried out at a field strength of 1–4 volts per centimeter for 10–30 hours, depending upon the mobilities of the components. The concentration of the proteins may be as high as 4%.

If for reasons of stability or solubility of components the isoelectric condition cannot be chosen, advantage can be taken of the difference in mobilities between the components. In this second mode of operation, all components are mobile and move toward the same membrane. However, the faster-moving components will be transported from the top compartment at a higher rate than the slower-moving components, resulting in a partial separation. The greatest separation of components is obtained by so choosing the operating time that half of the major component is transported out of the upper compartment.

In a mixture containing more than two protein components, the separation can be effectuated in steps, by first obtaining either the most acidic or the most basic component in pure state. In every step, the bottom cut is recycled several times, until substantially all isoelectric protein is separated; the pH is then readjusted to the isoelectric point of the second component and the process repeated until the mixture is resolved.

Another version of the apparatus was constructed by Raymond (58). His cell* consists of a length of dialyzing tubing, bent upon itself in U-shape. The bottom compartment is formed by the bottom of the U-shaped tubing, while both open ends of the casing serve as the top compartment. The intermediate portion of the casing is stretched in a plastic frame, forming two channels within which electrophoresis-convection takes place. Only this intermediate portion is exposed to the electric field. The claimed advantages of the cell are that the size of compartments can be varied at will by using longer or shorter lengths of casing. Moreover, there can be no leakage because the whole process is carried out within an intact length of tubing. Other modifications of the cell were proposed by Macheboeuf *et al.* (59) and by Tennent and Kniazuk (59a).

The bottom compartment containing the mobile components always also contains the isoelectric one at approximately its initial concentration, since the latter is not affected by the electric field. To obviate this shortcoming, a semicontinuous electrophoresis-convection apparatus was designed by Timasheff *et al.* (60). Buffer is continuously injected into the bottom compartment of a modified Cann apparatus at such a rate that the convective movement of the fastest component is at least as rapid as the upward flow of the total liquid, caused by the injection of the buffer. The top effluent of the apparatus is then passed into the bottom of another electrophoresis-convection cell, where the process is repeated. Such a cascading apparatus, with three channels in series, is illustrated in Fig. 10, and good separation of γ-globulin from serum and of mixtures of bovine serum albumin and bovine γ-globulin or deoxyribonucleic acid was achieved. Theoretical calculations, however, show that large volumes of buffer are required, resulting in considerable dilution of the fractions, while in practice, still greater volumes of buffer were found to be necessary, probably owing to the incomplete mixing of the injected buffer with the contents of the bottom compartments.

The theory of electrophoresis-convection was developed by Kirkwood and associates (61), utilizing principles of hydrodynamics. In solutions containing only one mobile protein component, the time t required to

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* E-C Apparatus Co., Swarthmore, Pennsylvania.

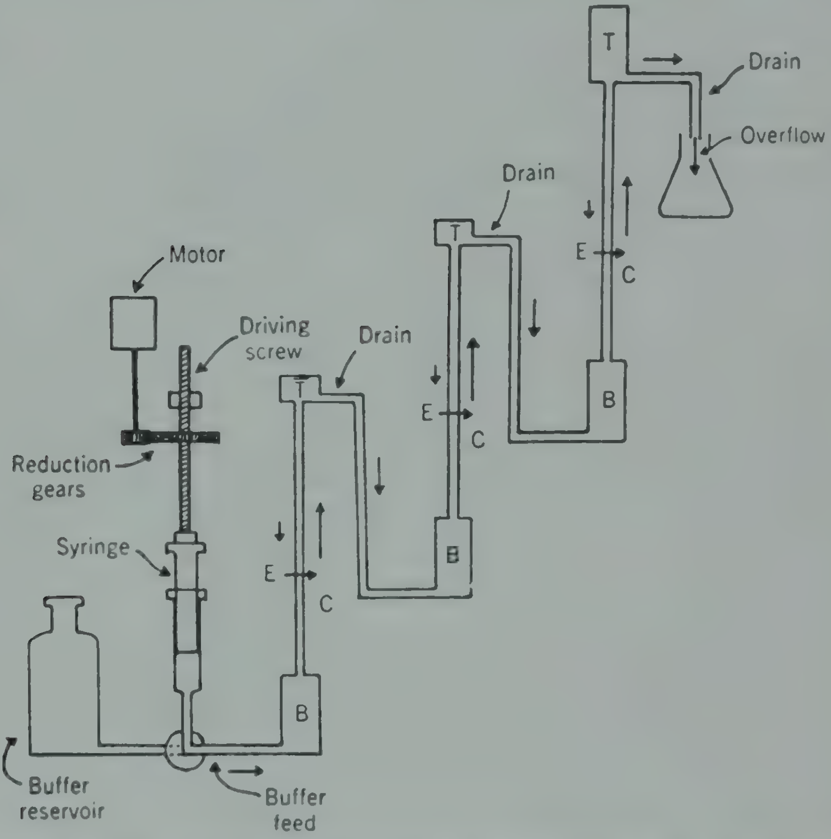
T
Drain
Overflow
Drain
T
E
C
Motor
Driving screw
Drain
T
E
C
B
B
Reduction gears
Syringe
E
C
B
Buffer reservoir
Buffer feed

FIG. 10. Schematic diagram of electrophoresis-convection with injection according to Timasheff *et al.* (60). Three cells are represented in series. *T*, Top reservoir; *B*, bottom reservoir; *C*, channel; *E*, electric field. The circulation of liquid is indicated by arrows drawn in the direction of flow. Each arrow is proportional to the rate of flow in the part of the apparatus next to it. Note that in the channels the rate of upward flow is considerably greater than that of the downward convective current.

transport a fraction $1 - \gamma$ of mobile protein from the top to the bottom reservoir is given by

$$t = \theta I(\gamma), \tag{1}$$

where

$$I(\gamma) = (5/3)^{5/4} \int_0^{1-\gamma} \{(1-\gamma)^{-3/5} - (1+\gamma)^{-3/5}\}^{5/4}\, d\gamma \tag{2}$$

and

$$\theta = 2VD/hblu^2E^2, \tag{3}$$

while

$$h = (4\eta lD/\alpha\rho gC_0)^{1/4} \tag{4}$$

The meaning of the symbols employed is as follows:

u, electrophoretic mobility of the protein
D, its diffusion constant
γ, the fraction of protein remaining in the top reservoir
C_0, the initial concentration of the protein in grams per 100 ml.
ρ, the density of the solvent
η, the viscosity of the solvent
b, channel width
l, channel length
V, volume of each reservoir
g, acceleration of gravity
E, electric field applied
$\alpha\rho$, the density increment produced by 1% protein

This theory was derived on the assumptions that the volumes of the top and bottom reservoirs are equal, that a laminar flow is maintained within the channel but that no net flow or liquid occurs vertically through it, and that the following limitations be satisfied:

$$uEa/D \ll 1, \; h/a \ll 1 \tag{5}$$

where a is the channel thickness.

The calculated values of the integral I, equal to t/θ, are presented as a function of $1 - \gamma$ in Table III.

TABLE III

TIME OF TRANSPORT AS A FUNCTION OF FRACTION OF MOBILE COMPONENT TRANSPORTED[a]

t/θ	$1 - \gamma$	t/θ	$1 - \gamma$
0.006	0.10	0.631	0.70
.029	.20	0.972	.80
.074	.30	1.57	.90
.145	.40	2.13	.95
.253	.50	3.21	.99
.407	.60	5.57	1.00

[a] According to Brown *et al.* (57).

Of the above two limitations, the second one is reasonably well satisfied in most experimental measurements, while the first one is usually not satisfied, the ratio uEa/D being usually greater than 1. The same equations apply, of course, to systems containing one mobile and one isoelectric component. The separation factors for two mobile components were also calculated, (61, 62), but as this method of separation is rarely employed, they will not be reported here.

The function θ has the dimensions of time, and by calculating it, it is possible, in theory, to predict the duration of the experiment necessary for certain desired transport of protein, making use of Table III. From equation (3), it would appear as if θ is inversely proportional to the product of the length l of the channel and b, its width, i.e. to the area of the cell. The thickness of the channel a does not appear in the expression, only in the limitations given above. The dependence of the time on the proportions of the cell is, however, contingent upon the mode of expressing the field strength E. In electrophoresis, it is often customary to express it in terms of $E = I/kA$, where I is the intensity of the current, k the specific conductivity of the solution, and A the cross section of the cell. In such a case, equation (3) becomes

$$\theta = 2VDk^2A/hu^2I^2 \tag{6}$$

and at constant amperage input I, the time required for separation becomes directly proportional to the area of the cell. From a practical point of view (63), it is more reasonable to express E in function of the power consumption in the cell P, since the latter gives the direct measure of heat generated and sets a limit to the field intensity that can be applied. The power consumption is given by $P = I^2R = I^2a/kA$ and equation (3) becomes

$$\theta = 2VDak/hu^2P \tag{7}$$

whereby it is apparent that the rate of separation of the components at constant power becomes completely independent of the dimensions of the cell except for the factor h, which contains the term $l^{1/4}$. The distance between the electrodes is, of course, the major factor in total power consumption in the entire apparatus, indicating the cooling necessary. For comparison of these conclusions with forced-flow electrophoresis, see page 298.

The above theory was tested on four proteins: carboxyhemoglobin, serum albumin, ovalbumin, and pepsin (57) under a variety of experimental conditions. The data were found to follow rather well the requirements of equation (3), provided that an empirical constant θ^* is employed instead of the calculated-time θ. The ratio θ^*/θ can be employed to calculate the ratio E^*/E of effective field strength to actual field strength

$$E^*/E = (\theta/\theta^*)^{1/2}$$

The ratio θ^*/θ varied between the extremes of 0.6 and 17, with an average of about 5, and it seemed to be rather constant for a particular set of experimental conditions. Essentially identical results were obtained in tests of the theory with separations of a mixture of two proteins, bovine albumin and globulin (62). This indicated that the separation is less effective than was predicted; this was attributed to a variety of factors including incomplete mixing in the top and bottom compartments, osmotic flow across the cell (inflow from the side of concentrated protein, outflow through the opposite membrane), and the fact that the limitation $uEa/D \ll 1$ was exceeded in most experiments.

B. Applications of Electrophoresis-Convection

1. Serum Proteins

Electrophoresis-convection was employed most extensively for the fractionation of serum proteins, starting either with the whole serum or purified fractions thereof. In a number of these studies, the aim was the determination of antibody distribution in the globulin fractions.

In the initial studies (5), the isolation of γ-globulin from the horse diphtheria antitoxin pseudoglobulin was attempted in phosphate buffer, pH 6.5, $\Gamma/2 = 0.1$. Under these conditions, the γ-globulin is isoelectric and, according to theory, best fractionation should result. The results were good, but far from ideal. Thus, fractionation of the original mixture (8% α-globulin, 56% β-globulin, 36% γ-globulin) for 10 hours at a field strength of 2 volts per centimeter yielded a top cut containing 56% γ-globulin. Refractionation of this top cut for another 14 hours at a field strength of 6 volts per centimeter increased the γ-globulin content in the top cut to 74%.

In a similar experiment utilizing bovine serum as the starting material (64), the isolation of γ-globulin was carried out under the above conditions while from the residual mixture, β-globulin was separated in phosphate buffer, pH 5.0 – 5.1, $\Gamma/2 = 0.3$, corresponding to its isoelectric point. By increasing the duration of the fractionation to 24 hours and the field strength to 3.1 volts per centimeter, a purification of γ-globulin from the initial 15% to 86% was achieved in the top compartment. A refractionation of this sample yielded a top cut containing 96% γ-globulin. The purest β-globulin fraction contained 20% albumin, 20% α-globulin, 1% β_1-globulin, 55% β_2-globulin and 4% γ-globulin.

In later studies involving the distribution of antibodies in sera, a more empirical approach to the fractionation was generally taken. In a typical experiment (65), each of the antisera was carried through seven successive stages of fractionation, the material from the bottom reservoir of the cell at the end of each stage serving as the starting material for the next, after dilution to initial volume. All fractionations were carried out in buffers of ionic strength 0.1 and at pH values ranging from an initial pH of 8.1 to a final pH of 5.3. Typical data are presented in Table IV, and it can be

TABLE IV

Fractionation of Human Serum[a]

Fraction	pH Fractionation	Buffer	Apparent concentration of electrophoretic components				Mean mobility of gamma
			Albumin	Alpha	Beta	Gamma	$-10^5 \times \bar{u}$
Serum III:			71	15	5	9	1.36
Top-1	8.10	Phosphate	—	—	—	98	0.94
Top-2	7.43	Phosphate	—	—	1	99	1.25
Top-3	6.95	Phosphate	—	—	3	97	1.47
Top-4	6.45	Phosphate	—	—	11	89	1.55
Top-5	5.95	Phosphate	—	—	36	64	1.80
Top-6	5.45	Phosphate	3	4	71	21	1.86
Top-7	5.32	Acetate	9	44	41	6	2.24
Serum IV:			69	10	11	9	1.29
Top-1	8.10	Phosphate	6	—	7	87	0.90
Top-2	7.49	Phosphate	2	—	4	94	1.27
Top-3	7.01	Phosphate	3	—	7	90	1.52
Top-4	6.50	Phosphate	1	—	7	92	1.54
Top-5	6.00	Phosphate	9	4	41	46	1.93
Top-6	5.50	Phosphate	9	18	36	37	2.06
Top-7	5.32	Acetate	16	22	47	15	2.37

[a] According to Cann *et al.* (65).

readily seen that the top fractions obtained at pH 8.1 and 7.5 contained essentially only γ-globulin. The results at these pH values are substantially better than those obtained at pH 6.5, although the latter corresponds to the mean isoelectric point of the γ-globulin. The reasons for the better performance under conditions where the bulk of the γ-globulin was not isoelectric but was moving in the same direction as the rest of the proteins was not explained, but the data are consistent with a whole series of other reports, as well as with the reviewer's own observations in forced-flow electrophoresis. The last column of Table IV reports the mean mobility of the γ-globulins in the different fractions. It indicates that a subfractionation of the γ-globulin fraction was also achieved. While the proportion of other proteins varies in the various fractions collected, no other component was obtained in pure state through electrophoresis-convection alone. Salt fractionation was therefore employed for the isolation of the serum albumin.

Starting with a purified γ-globulin fraction, it was possible to separate it into a total of eight fractions with constituent mean mobilities ranging from -1.25 to -2.25×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, analyzed in the usual barbiturate buffer, pH 8.7, and mean isoelectric points ranging from pH 7.31 to 5.74, by subjecting it to successive fractionations in phosphate buffers, the pH of which was varied from pH 7.60 to 5.48 (66). In another experiment in which four fractions were obtained, all four were found to have a Gaussian distribution of mobilities, the addition of these coinciding with the distribution in the original sample (67). The ultracentrifugal analysis of γ-globulin prepared by electrophoresis convection revealed the absence of all heavy components found in the γ-globulin prepared by the ethanol fractionation technique (68, 69). The observed value of the mean sedimentation constant $s = 6.56\ S$, together with a diffusion constant of $D_{20,w} = 3.84 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, gave a molecular weight of 162,000, assuming a partial specific volume of 0.745. Detailed analysis of the sedimentation pattern has shown that the γ-globulin is heterogeneous, but no correlation with the electrophoretic heterogeneity was observed.

In human antipertussis γ-globulin, the protective activity was associated to a higher degree with the slowest moving fraction (in barbiturate buffer), although present in all fractions (70). Similar fractionation of γ-globulin in rabbit antisera containing antibodies against phenyl arsonate and against bovine globulin resulted also in partial separation of the antibodies, demonstrating that different antibodies are characterized by different electrophoretic mobilities (71). In human Rh-antisera, it was found that the Rh antibodies were distributed throughout the serum globulins including the α-globulins, the quantitative distribution between the fractions varying with the blood type and also with the individual (65, 72).

A number of sera obtained from individuals highly allergic to insulin (73–76), ragweed (75, 77) and cottonseed pollen (76, 77), were also fractionated. It was found that the sensitizing antibody activity was associated with a fraction immobilized in the top cut at pH values between pH 6 and 5.3, this fraction being particularly rich in β-globulin, while the blocking activity was associated with γ-globulin (faster moving). A curious difference was observed in the distribution of the antibodies in sera of polio-immunized humans and monkeys. The antibodies in humans were found to be associated with γ-globulin, while in monkeys, they were associated with the β-globulin (78). In a fractionation of antisera to the bacteriophage T2r^+, the activity was associated with γ-globulin, though association with other fractions could not be excluded (79).

2. *Other Proteins*

A number of egg white proteins were fractionated by electrophoresis-convection. Cann and Kirkwood (80) first reported on the separation of the components of ovalbumin. Another application was the isolation of conalbumin by Phelps and Cann (81) and Timasheff and Tinoco (81a). The usual preparation by ammonium sulfate precipitation from acid solutions results in an irreversible modification of this protein, which was avoided by carrying out the electrophoretic separation above pH 4.5.

The potentialities of electrophoresis-convection for the separation of complex mixtures of very similar proteins are exemplified by the fractionation of ovomucoid by Bier *et al.* (82, 83). A total of five distinct components is detectable on electrophoretic analysis in buffers of low ionic strength, their isoelectric points being: O_1, pH 4.41; O_2, pH 4.28; O_3, pH 4.17; O_4, pH 4.01; and O_5, pH 3.83. Upon repeated fractionations by electrophoresis-convection at low ionic strength, a preparation was obtained containing O_1 as the only electrophoretically detectable component. Several other fractions contained increased concentrations of components O_2 and O_3. The data on these fractions are presented in Table V and some characteristic diagrams are presented in Fig. 11. These data clearly indicate that at least the major components of the ovomucoid

TABLE V

CHARACTERIZATION OF OVOMUCOID AND ITS FRACTIONS[a]

Sample[b]	Approximate percentage of components[c]					Sedimentation constant $s_{20,w} \times 10^{13}$	Hexoses (%)	Glucosamine (%)	Antitryptic activity[d]
	O_1	O_2	O_3	O_4	O_5				
Unfractionated	30	45	17	6	2	2.41	8.2	13.0	7.0
Fraction 1	100					2.36	8.3	13.1	7.8
Fraction 2	85	10	5			2.53	—	—	7.4
Fraction 4	5	20	45	25	5	2.47	9.9	13.3	7.9
Fraction 6	12	73	15			2.34	8.7	13.5	6.8
Fraction 7	10	60	20	7	3	2.37	8.9	14.4	7.3

[a] According to Bier *et al.* (83).

[b] Fractions refer to the fractionation scheme (83).

[c] Area determinations permitted only an approximate estimation of the relative ratios of components.

[d] Activity of lyophilized egg white taken as 1.

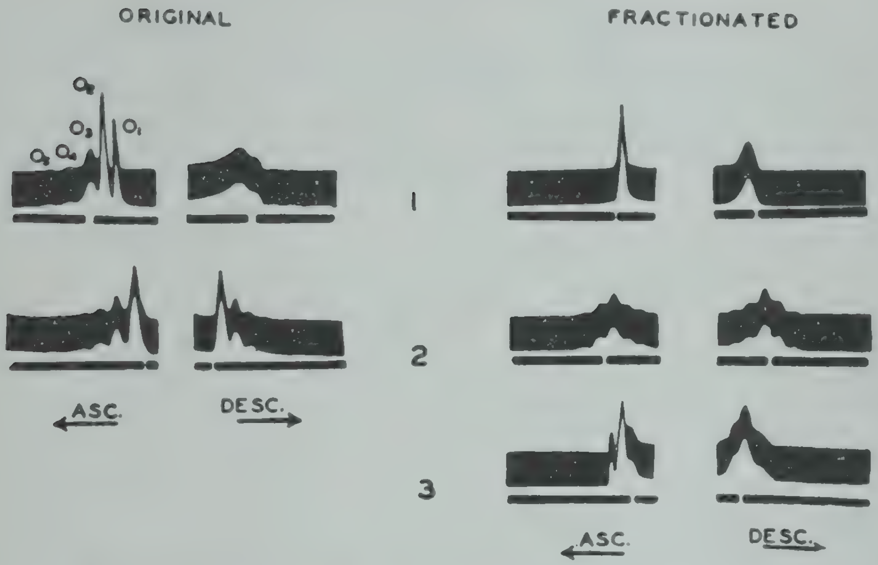

FIG. 11. Electrophoretic patterns of ovomucoid. From Bier *et al.* (83). Original ovomucoid: *1*. pH 4.13; *2*. pH 4.49. Ovomucoid fractionated by electrophoresis-convection: *1*. pH 4.49, fraction 1; *2*. pH 4.13, fraction 4; *3*. pH 4.13, fraction 6.

system, although electrophoretically distinct, are endowed with comparable biological activity as trypsin inhibitors. Similar identity of biological activity in electrophoretically distinct proteins was encountered in trypsin (84, 85).

Insulin is another example of an electrophoretically heterogeneous protein which could be separated by electrophoresis-convection. The data of Timasheff *et al.* (86) are reproduced in Table VI and clearly show that a partial resolution was attained. This is somewhat surprising in view of the chemical evidence for the homogeneity of insulin. It raises the problem of whether electrophoretic inhomogeneity is necessarily due to chemical inhomogeneity, although such a relationship was proved at least in the case of the ovalbumin components (87). This problem of microheterogeneity of proteins is discussed at greater length by Brown and Timasheff in Chapter 8 of this book.

The possible application of electrophoresis-convection to the purification of enzymes is exemplified by the work of Mathies (88), who achieved a threefold purification of the swine kidney alkaline phosphatase by repeated refractionation of the top cuts. The final product was reported to be twice as active as the best previously available preparation. Cann and Frisell (89) succeeded also in partially purifying D-amino acid oxidase from extracts of pig kidney cortex. Best results were obtained at pH 7.0, where the top fraction had 3–5 times the specific activity of the extract.

Singer *et al.* (90) have suggested that by complexing proteins with

TABLE VI

FRACTIONATION OF INSULIN IN pH 7.5 PHOSPHATE ($\Gamma/2 = 0.1$)[a]

Sample	Conditions	Fraction	Volume (ml.)	Conc. (mg./ml.)	Activity (units/mg.)	Mobility (cm./sec./v./cm. × 10^5)
Zn-cryst.[b] lot #515499 Lilly	1.9 v./cm., three 12-hr. stages	Starting	280	5.7	27	−4.85
		Pooled tops	432	0.4	16	−4.58
		Final bottom	156	7.1	25	−4.86
Recovery: protein, 82%; activity, 71%. Hyperglycemic factor present in all fractions.						
Amorphous #200-1B-11J Lilly	2.1 v./cm., 13 hr.	Starting	125	6.5	24	−5.06
		Top	52	0.17	15	−4.22
		Bottom	78	11.0	23.5	−5.10
Recovery: protein, 106%; activity, 105%. Hyperglycemic factor present in all fractions.						

[a] According to Timasheff *et al.* (86).

[b] Electrophoretic analyses carried out on the Klett instrument.

[c] Electrophoretic analyses carried out on the Perkin-Elmer instrument.

appropriate polyelectrolytes, higher transport rates can be achieved. This was substantiated with synthetic mixtures of bovine serum albumin and deoxypentosenucleic acid. Timasheff and Kirkwood (91) have applied the same procedure to the fractionation of insulin-protamine complexes, confirming the electrophoretic heterogeneity of insulin.

V. Forced-Flow Electrophoresis

A. Principles and Methods

Electrophoresis-convection is an elegant preparative method capable of very fine resolution. In the course of a fractionation the two fractions separated in the channel are constantly remixing with the original solutions in the top and bottom reservoirs. As a result, the composition of the solutions in these two compartments changes by a process of progressive dilution with the two fractions as they emerge from the channel, and asymptotically reaches the composition of the latter. It would seem, therefore, that the separation finally achieved can never be better than that obtainable on a single passage through the channel.

A method in which the two fractions are separated immediately upon leaving the channel was proposed by Bier (7, 92). It is illustrated by the diagram presented in Fig. 12. The electrophoretic cell consists of two outer semipermeable membranes *A* and *A′*, held stretched in a Plexiglas frame (not shown in the diagram). The cell is immersed in a circulating cooled buffer, and a direct-current electric field can be established across the membranes. The two membranes are kept parallel, 3 to 4 mm. apart, while a third semipermeable membrane *B* is inserted between them, part way into the cell. The cell is thus divided into three compartments, two at the top and a common one at the bottom. All three compartments have means for continuous input or withdrawal of the colloid solution.

A projected view of the components of the cell is presented in Fig. 13. The shaded surfaces represent the three membranes, the center one having a window cut out, which defines the size of the common bottom compartment. From both sides of the center membrane there are two spacers, determining the size of the whole cell. Although there is an advantage in keeping these spacers as thin as possible, a minimum thickness is necessary to permit the introduction and withdrawal of the liquid through the spacers by means of embedded stainless-steel tubing connecting with a manifold system that distributes equally the flow of the liquid over the entire width of the cell. The spacers and membranes are held between two outer frames which are tightened together by nylon screws. Platinum electrodes, external to the cell and coextensive with it, establish the electric field. Surgical polyethylene tubing, available in graded sizes corresponding

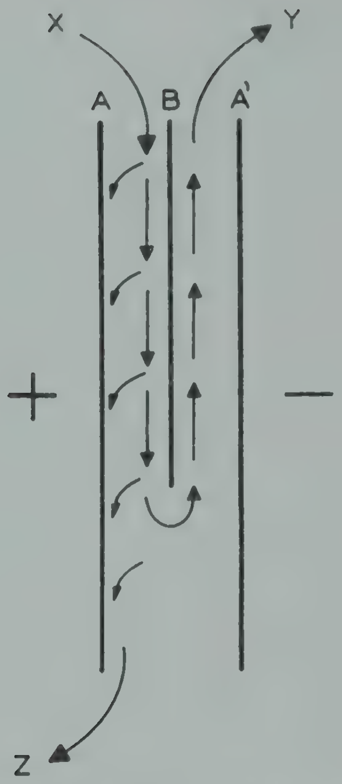

FIG. 12. Schematic presentation of the forced-flow electrophoresis cell, according to Bier (7). *A*, *A′*, outer membranes; *B*, intermediate membrane with cutout; *X*, input of solutions; *Y*, *Z*, outlets for separated fractions.

to the caliber of the stainless-steel tubing (No. 18), serves to connect the cell with the supply bottle of the solution to be fractionated, and with the receiving bottles for the two fractions.

Reverting to the schematic presentation of Fig. 12, it is easy to describe

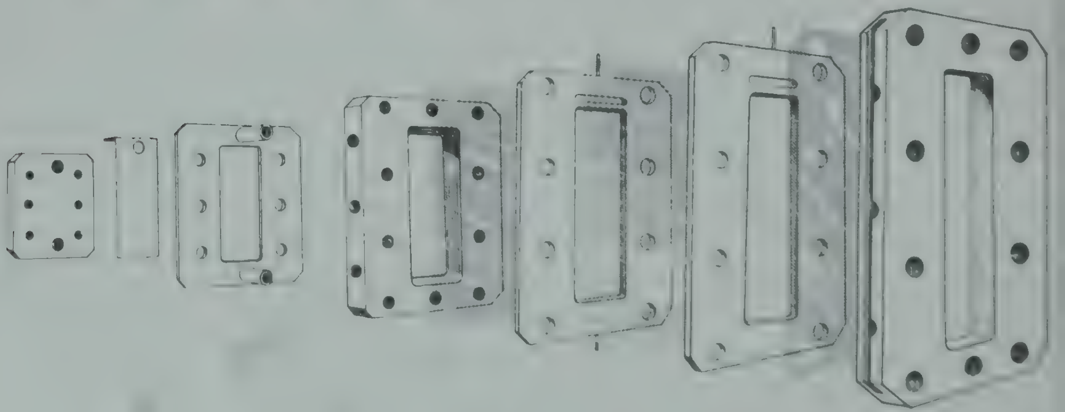

FIG. 13. Projected view of the forced-flow electrophoresis cell. (Courtesy of Perkin-Elmer Corp., Norwalk, Connecticut.) Shaded areas represent membranes. Only one electrode assembly is shown, at the extreme left.

the operating principle of the apparatus. If it is assumed that the solution to be fractionated contains two proteins, differing in their isoelectric points, then the pH of the solution and of the outside buffer is adjusted close to the isoelectric point of one of the proteins. The solution is continuously fed into one of the top compartments, and the polarity of the current is so selected that the mobile protein migrates toward the outer membrane of the compartment, as indicated by the short arrows in Fig. 13. This migrating protein will have formed a layer of increased concentration in the immediate vicinity of the outer membrane and will be withdrawn quantitatively in that part of the liquid which is collected through the bottom compartment. The other protein, at or close to the isoelectric point, will not have been influenced by the electric field and will be uniformly distributed throughout the liquid.

The inflowing liquid is thus separated into two fractions. The bottom fraction contains all the migrating proteins, but is still contaminated with the isoelectric protein in its original concentration. The second fraction, obtained at the top of the cell, contains only the isoelectric protein in a high state of electrophoretic purity, with all the mobile protein eliminated. The method has the added advantage that the rates of withdrawal of the solutions from the top and bottom of the apparatus can be controlled independently, the flow through the top outlet being adjusted, for example, to four times the flow through the bottom outlet. Accordingly, in a single circulation of the solution through the apparatus, it is possible to obtain 80% of the isoelectric protein at its original concentration, while all of the mobile component is obtained fivefold concentrated, admixed with only 20% of the initial amount of the isoelectric protein. If greater recovery of the isoelectric component is desired, the bottom fraction can be recirculated. The method can thus be used either for the preparation of electrophoretically pure proteins or for the concentration of protein solutions, as may be desirable, for example, in highly dilute, enzymically active, metabolic filtrates.

B. Theoretical Considerations

The method as outlined above, has some resemblance to continuous electrophoresis-convection. This resemblance is merely apparent, since different operative principles are involved. Both methods obviously depend upon the laminar flow of the solutions in the cell, as well as upon the horizontal transfer of the colloids through electrophoresis. However, the present method does not depend upon the gravity-caused convection of the fluid in the cell for the separation of the components. Rather, the geometry of the cell imposes a flow pattern on the liquid. Thus, it is also operative in a horizontal position, which is certainly incompatible with electrophoresis-convection. In its preferred position, the vertical, the flow pattern is so adjusted, however, that the convection proceeds in the same direction as the forced flow of the liquid, thus contributing to the efficiency of the system. This convective effect is by no means negligible, and it explains why the cell does not operate reversibly, i.e. it is not possible to obtain equally good separation if the polarity of the electric field is reversed and one attempts to withdraw the mobile component through the top outlet Y.

It is easy to formulate an idealized theory for the performance of the apparatus (92a). If d is the distance separating the outer membrane from the center one, and l is the length of the center membrane, then good separation will take place if the mobile component will electrophoretically travel the distance d in the time t required for the liquid containing it to flow from the input to the bottom of the center membrane, i.e. flow over the length l. By introducing the usual expression $E = I/k\,l'w$, relating the electric field to the specific conductance k and cross-area $l'\,w$, l' being the over-all height of the cell, w its width, the following equation is obtained:

$$v = I\,\frac{u}{k}\,\frac{l}{l'} \simeq I\,\frac{u}{k} \tag{8}$$

where v is the rate of outflow of protein solution through outlet Y.

Accordingly, the rate of separation obtainable with a cell is independent of all cell dimensions except for the ratio l/l' and depends only upon the mobility of the mobile component, the amperage applied, and the conductivity of the solution, low ionic strength favoring high flow rates. The reason is obvious, since there is a direct relation between the time of residence of a given volume of liquid in the cell and the cell dimensions. Moreover, practice has shown that the ratio l/l' is also not critical, for reasons that will be shown below and can be approximated as being equal to 1. This conclusion differs from equation (3) obtained for electrophoresis-convection, demonstrating that different principles are operative.

Taking as an example $u = 5 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}, $k = 2 \times 10^{-3}$ reciprocal ohm, $I = 1$ amp., a flow rate of about 2.5×10^{-2} ml. per second, or 1.5 ml. per minute is calculated, which is of the order of magnitude obtainable in actual separations. The above treatment, however, is at best only a gross approximation of reality. Its primary shortcoming is that it presupposes a gravitationally stable system in the cell. Actually, the system is gravitationally unstable in both the horizontal and vertical direction. In the horizontal direction, the accumulation of the protein at one of the membranes will set up a certain convective flow, similar to that in electrophoresis-convection. The vertical stability is due to the fact that the protein solution is continuously being fed on top of a solution of lesser protein concentration, some of the protein having already migrated toward the membrane. Therefore, it will sink into the underlying liquid at a certain rate which is quite independent of the over-all rate of flow of the liquid through the cell.

As a result of these factors, under proper operating conditions, a definite flow pattern is established in the cell, dependent upon a large number of variables, such as rates of inflow and outflow, temperature of inflowing liquid versus that of the cell content, temperature gradients within the cell, density increment due to protein content, field strength, etc. This also explains the experimental observation that the proportion between flow rate and amperage input, which is expected from equation (8), is actually observed only over a limited range of these two variables, as shown in Section V, D (Table VIII). The problem appears to be far too complex to permit the formulation of a more sophisticated theory which would take into account all these factors. Moreover, it is doubtful whether there would be better agreement with experimental results, because of purely geometrical factors in the cell design, such as the construction of inlet and outlet manifolds, bulging of membranes, etc.

For proper design of a cell, consideration should be given to the power consumption, not only to the total amperage. While the over-all distance between electrodes will largely govern the total heat generated within a system, at present only the heat generated within the cell itself will be considered. The power P in watts used up in the cell is given by

$$P = I^2 R = I^2 d/k\,wl \tag{9}$$

where I can be calculated from equation (8). The heat in calories per second generated within the cell is 0.24 times the power P. The above equation (9) provides a simple measure of estimating the actual feasibility of a separation and the requirements of cell design. As an example, data are presented in Table VII showing the watts used and calories per minute

TABLE VII

POWER AND HEAT RELATIONS IN FORCED-FLOW ELECTROPHORESIS[a]

	$u = 10^{-5}$cm.^{2}v.$^{-1}$sec.$^{-1}$		$u = 5 \times 10^{-5}$cm.^{2}v.$^{-1}$sec.$^{-1}$	
Cell area (cm.^{2})	Power (watts)	Heat (cal./min.)	Power (watts)	Heat (cal./min.)
10	167.0	2400	6.7	96.0
100	16.7	240	0.67	9.6

[a] Flow rate = 1 ml./min.; conductivity buffer = 2×10^{-3} reciprocal ohms; distance between membranes = 0.3 cm.

generated in two cells of cross-areas 10 and 100 cm., respectively, distance $d = 0.3$ cm., in a buffer of conductivity $k = 2 \times 10^{-3}$ reciprocal ohms, for two hypothetical proteins of mobility $u = 10^{-5}$ and 5×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, if flow rates of 1 ml. per minute are desired.

A flow of 1 ml. per minute being assumed, the calories per minute given in Table VII correspond to the rise in temperature in °C. of the solution passing through the cell, if no heat exchange takes place across the membranes with the outside buffer. It is obvious that only the last example is possible without external cooling, the expected temperature rise being 9.6°C. Separation could then be carried out in multicell units without cooling spaces interposed. The first case is clearly impossible under all cooling conditions. The two intermediate cases necessitate rapid circulation of cooled buffer around both sides of the cell, for proper heat exchange.

C. Filter Paper Modification

An alternate procedure of employing the cell described in Figs. 12, 13 was developed by the author. In trying to find the best way for withdrawing the separated fractions, various other arrangements of the center membrane were studied. These included center membranes extending over the entire length of the cell and having openings of various geometrical patterns, such as vertical or horizontal slits, perforations, etc. Moreover, various fully permeable membranes were studied, such as cotton, silk, and nylon cloth.

Only one arrangement resulted in an improved performance of the apparatus. This was the substitution of the center membrane of Fig. 12 with a hardened wet-strength filter paper, Whatman No. 52 or 54 being satisfactory. This insertion separating the cell into two compartments, various flow patterns are possible, but in the preferred arrangement, the inflow and outflows are as previously described, i.e. the protein solution is introduced through the top of one of the compartments, the polarity so adjusted that the mobile component migrates toward the outer membrane of the same compartment and is withdrawn at the bottom. The rest of the solution, containing only the isoelectric component, is withdrawn from the top of the other compartment. The filter paper has sufficient resistance to the flow of liquid to force a uniform flow across its entire surface. The fraction containing the isoelectric protein is therefore uniformly withdrawn from the entire cell cross section.

Considering this simplest mode of operation, the theoretical requirement for proper separation is that the rate of flow of liquid through the filter paper be less than the rate of electrophoretic migration of the mobile component in the opposite direction. By equating the two rates, one obtains

$$v = uEA = I\,\frac{u}{k} \tag{10}$$

This equation is identical with the equation (8), except for the ratio l/l'. In practice, this method was found to produce better results than the previous one. The striking advantage is that in this mode of operation, the proportion between rates of flow and amperage imput were found to be followed over the entire experimental range studied.

The filter paper modification has certain similarities with the so-called compartment type of electrophoretic instruments. Many other authors have designed compartment-type cells, most of them having been reviewed in detail by Svensson (1). They will not be considered here since the reviewer is not aware of any recent significant contributions to this field.

The instruments having the greatest similarity to the above-described cell were the two cells designed by Theorell (93–95) and an apparatus of Hahn and Tiselius (96). One of the Theorell cells is presented in Fig. 14, and the Hahn and Tiselius apparatus in Fig. 15. Both instruments have received limited application only, Theorell having utilized it to purify the yellow enzyme, while Hahn and Tiselius have briefly reported on the purification of pepsin and hyaluronidase. Therefore, the instruments are mainly of historical interest and the reader is referred to the original literature for the description of the cells and their use. In either cell, no provision was made for efficient heat dissipation, and therefore only very small field intensities were employed and the operation was batchwise, each step requiring many hours.

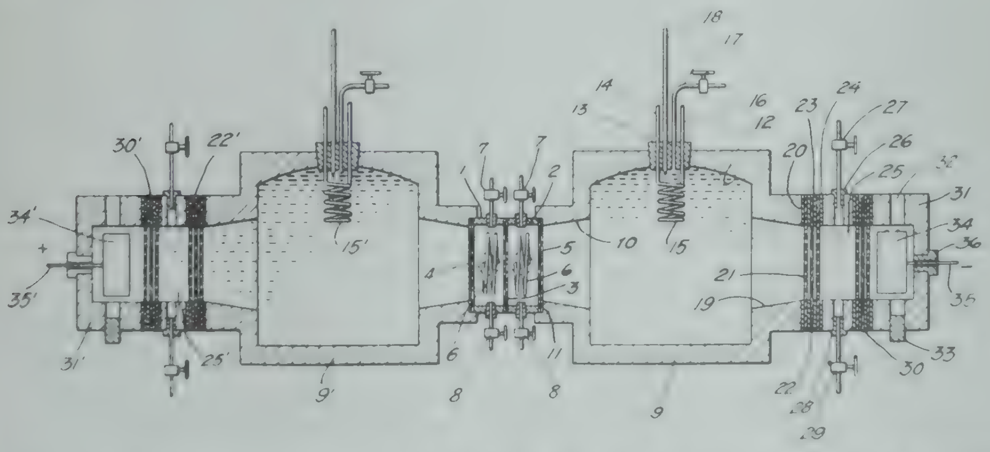

FIG. 14. Compartment-type cell of Theorell (94, 95). *34*, *34′*: electrodes; *9*, *9′*: intermediate buffer compartments; *4*, *5*: semipermeable membranes; *3*: fully permeable membrane.

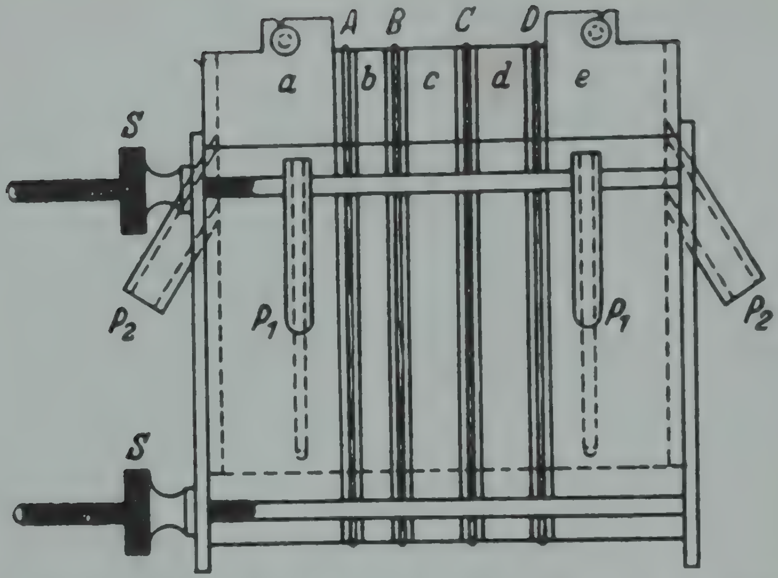

FIG. 15. Compartment-type cell of Hahn and Tiselius (96). *B*, *D*: semipermeable membranes; *C*: fully permeable membrane; *a*, *e*: electrode compartments.

Obviously, there is great similarity between the two versions, the window-type and the filter paper of the Bier apparatus. This similarity includes the following points: (a) the apparatus is identical; (b) the same arrangement of inflow and outflows can be utilized; (c) equations (8) and (10) are comparable; (d) experimental results are of the same order of magnitude. This appears to be disturbing since these two arrangements are the most efficient (in terms of flow rates obtainable and field strength applicable) prototypes of two types of electrophoretic methods that are usually considered to be quite distinct, namely, the compartment and the convection methods (1). However, it is explained by the fact that the high rates of flow obtainable with the filter paper arrangement are due precisely to a combination of the two methods, the cell being operated in such a way that advantage is taken of the convective flow to increase the rate of separation. Moreover, it is possible to provide the filter paper membrane with a window at the bottom, as described in Fig. 12, thus passing continuously from one to the other type of operation. It is therefore doubtful whether the above distinction remains necessary, and it is for this reason that the term "forced-flow electrophoresis" is suggested for those methods of continuous electrophoresis without supporting media in which the construction of the cell forces on the solution a certain desired flow pattern, so as to effectuate the physical separation of fractionated components. The apparatus recently described by Dobry and Finn (96a) belongs into this category, as it employs a forced laminar flow stabilized by the addition of dextran or Methocel. While not yet tested on proteins, it offers promise of being suitable for the simultaneous recovery of more than two fractions.

D. Applications of Forced-Flow Electrophoresis

The performance of a number of cells of different dimensions and under a variety of experimental conditions was studied by Bier (92a). Having a dual aim of optimizing cell design and studying the principles involved, most of this work was carried out with a single system: a 0.3% human carboxyhemoglobin solution in 0.02 *M* barbiturate buffer, pH 8.6. The hemoglobin was the mobile component, having in this buffer a mobility of about 4.8×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, the conductivity of the buffer being 0.74×10^{-3} reciprocal ohms. No isoelectric component was included, and the results were reported in terms of per cent residual hemoglobin present in the top effluent of the cell. Its concentration was estimated colorimetrically, perfect separation of the mobile component resulting in colorless effluent through outlet *Y*.

The dependence of residual concentration on rates of total flow through the apparatus, with equal rates of withdrawal through both outlets, is presented in Fig. 16 as a function of current intensity. Data for vari-

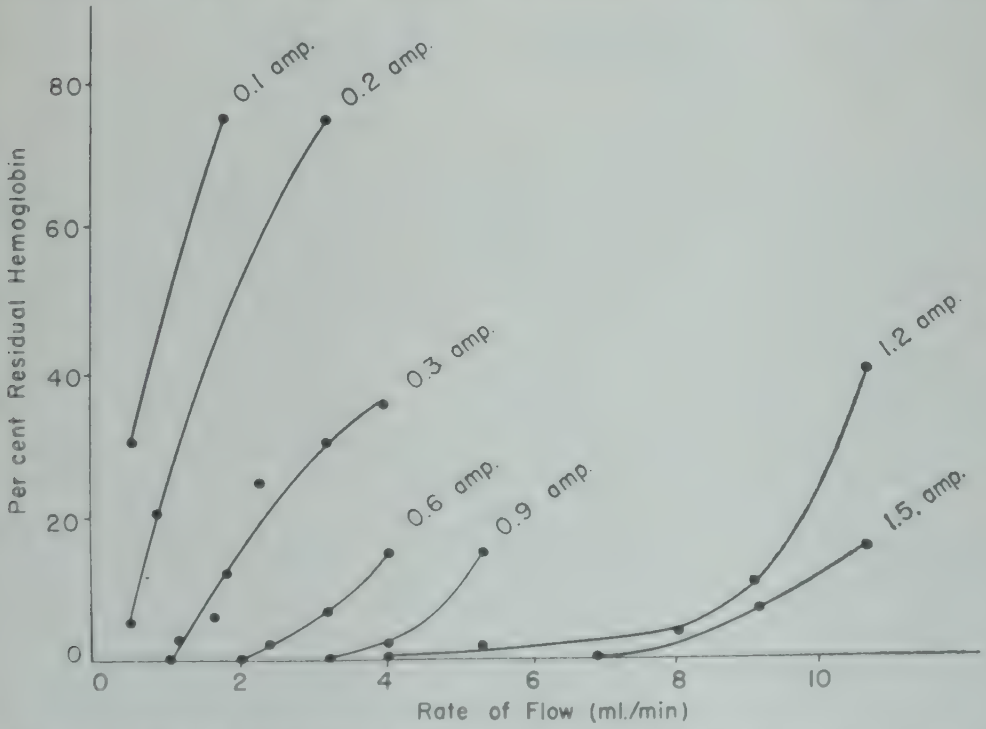

FIG. 16. Rate of hemoglobin transfer by forced-flow electrophoresis (103). Cell dimensions, 7.5 × 1.9 cm.; Whatman No. 52 center membranes; buffer, sodium barbiturate, pH 8.6, $\Gamma/2 = 0.02$.

ous cells and center membranes are summarized in Table VIII, giving the interpolated flow rates for 10% residual hemoglobin concentration at different current intensities. The rate of separation increases with the porosity of the filter paper membrane, Whatman No. 54 giving best results. The rate of separation is consistently higher with the filter paper than with the window-type center membrane, though both are of the same order of magnitude. The difference is largely due to the fact that with the window type membrane, the flow rates do not parallel the current intensities applied. This is particularly noticeable with the smallest cell tried, and its causes are discussed in the preceding Section B. The cross-areas of cells used were 14.25, 67.5, and 100 cm.^{2}, respectively, yet the flow rates were of the same order of magnitude, in accordance with equations (8 and (10). The smallest cell gave significantly poorer results only with the window-type arrangement. An exact comparison of the three cells is difficult, due to the inherent scattering of experimental values. Moreover, up to 20% of current bypasses the cells through the surrounding buffer and, thus, the field is smaller than calculated. However, an important effect of cell size has to be emphasized, i.e. with the larger cells proportionately smaller voltages are operative, causing less heating within the cell, in accordance with Table VII. In the smallest cell, using the filter paper membrane, strong electroosmotic effects were also noted at highest current intensities. Both these factors show the larger cell to be more advantageous in many applications.

TABLE VIII

RATES OF FLOW IN MILLILITERS PER MINUTE FOR 90% CARBOXYHEMOGLOBIN TRANSPORT BY FORCED-FLOW ELECTROPHORESIS

		Current in amperes									
Cell	Center Membrane	0.2	0.3	0.4	0.6	0.9	1.0	1.2	1.5	2.0	2.4
7.5 × 1.9 cm.	Whatman 54			3.0	5.0	6.3					
7.5 × 1.9 cm.	Whatman 52	0.6	1.8		3.5	5.0		9.1	9.5		
7.5 × 1.9 cm.	Window			1.3	1.3	1.3					
15 × 4.5 cm.	Whatman 54		2.0		3.6		7.0				
15 × 4.5 cm.	Whatman 50		0.8		2.4		4.3				
15 × 4.5 cm.	Window						3.2		3.9	4.0	5.3
15 × 4.5 cm.	Window in Whatman 54				3.8		5.3				
10 × 10 cm.	Window					2.7		3.0	3.2		

Separation of γ-globulin from half-diluted human serum was carried out in veronal buffer, pH 8.6, Γ/2 = 0.02, and current intensity of 1.2 amp in the 15 × 4.5-cm. cell, using a window-type membrane. At a rate of flow of about 2 ml. per minute, a top fraction was obtained showing, on electrophoretic analysis, only γ-globulin. By maintaining the same conditions but changing the outflows through the top and bottom outlets to a ratio of 4:1, a slight contamination with β-globulin was observed. The two diagrams are presented in Fig. 17. This separation was obtained in a single passage through the cell. A serial fractionation of human serum was carried out in phosphate buffers, 0.02 *M*, of progressively decreasing pH values. At each pH value, the serum was twice fractionated, and the bottom effluents were always dialyzed against each successive buffer prior to fractionation. The composition of the resulting top fractions is presented in Table IX. Whole blood, i.e. plasma as well as the corpuscular elements, were also subjected to fractionation, and the results are summarized in the following Section E.

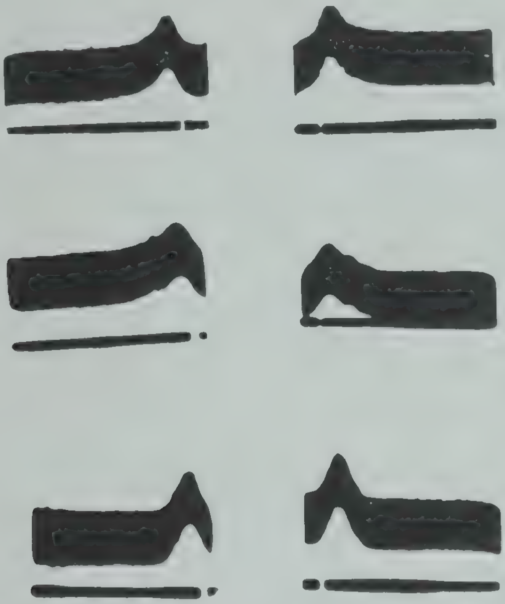

Fig. 17. Diagram of γ-globulin prepared by forced-flow electrophoresis, at pH 8.6, barbiturate buffer (92a), from half-diluted human serum. Top diagram: flow rate of 2 ml. per minute, at equal rates of outflow of the two fractions. Center diagram: rates of outflow through top and bottom kept in ratio of 4:1. Bottom diagram: 0.5% solution of Armour bovine γ-globulin.

Seibert (97) has applied forced-flow electrophoresis for further purification of well-characterized polysaccharides of tubercle bacilli. Some of the results are presented in Table X. Striking is the complete separation of the type I from type II polysaccharide, obtained on single passage through the cell of the fraction 107-III. These polysaccharides are important in the

TABLE IX

FRACTIONATION[a] OF SERUM BY FORCED-FLOW ELECTROPHORESIS

Fraction	Albumin	α_1	α_2	β	γ
Original	59.5	4.0	9.2	11.1	16.2
Top, pH 8.2	4.3	2.2	2.7	12.0	78.8
Top, pH 7.7	0	0	0	2.2	97.8
Top, pH 6.6	0	0	0	33.0	67.0
Top, pH 6.2	55.5	6.7	8.6	12.5	16.7
Top, pH 5.8	69.5	4.7	8.5	11.0	6.2

[a] Fractionation in 0.02 *M* phosphate buffer, flow rate 2.7 ml. per minute, current = 1.2 amp., 7.5 × 1.9-cm. cell, Whatman No. 54 center membrane.

TABLE X

SEPARATION AND PURIFICATION OF TUBERCULOPOLYSACCHARIDES BY FORCED-FLOW ELECTROPHORESIS[a]

Polysaccharide fraction	Cycle	Field strength (volts/cm.)	Flow rate (1 ml./min.) Top: Bottom	Percent of total as:		
				Polysaccharide		
				Type I	Type II	Protein
107 III	Original			57.9	28.4	13.6
	1st (Pooled)	11–44	1:4	91.0	0	9.3
	2nd	22	1:6	92.0	0	7.9
	3rd	22	1:4	94.3	0	5.7
	3rd (2)	22	1:4	95.5	0	4.6
S 4	Original			93.9	0	6.1
	1st	44–55	1:5	97.0	0	3.1
	Original			96.6	0	3.3
	1st (1)[b]	33	1:4	96.7	0	3.3
	1st (2)[b]	33	1:6	96.2	0	3.9
	1st (3)[b]	33	1:6	96.5	0	3.5
S 6[c]	Original			91.8	7.0	1.2
	1st (1)	55	1:5	84.2	15.3	0.5
	1st (2)	55	1:6	78.6	20.6	0.85

[a] According to Seibert (97).

[b] Buffer in all cases was phosphate pH 7.0, $\mu = 0.01$ except in cases starred where it was borate buffer pH 9.2, $\mu = 0.01$.

[c] The top effluent in these cases was at the positive pole; in all other cases it was negative.

immunology of tuberculosis, type II being antigenic, while type I is a hapten, able to interfere with complement fixation. The elimination of protein contaminants attempted in subsequent cycles was not as successful. The technique for cycling was guided by the color of the solutions. The original solutions were a very deep or moderately deep amber color, and satisfactory separation was obtained only when the top effluent became colorless. Relatively small flow rates were employed, the work having been done with the smallest cell and a window-type membrane; this combination was shown in Table VIII to be the least effective.

Worthington Biochemical Laboratory (98) has adopted forced-flow electrophoresis for the preparation of two highly purified enzymes: elastase and horse-radish peroxidase. Elastase is first crystallized according to Lewis *et al.* (99), resulting in a product which is highly insoluble, having an activity of 15–20 units per milligram, as assayed according to the method of Sachar *et al.* (100). The crystalline material is electrophoretically inhomogeneous at several pH values, containing a slow-moving major and a fast-moving minor component. Separation in the Aminco apparatus has shown that only the slower component is enzymatically active and, if separated, is highly soluble, with an isoelectric point at pH 9.5 (99). The same conditions were employed in forced-flow electrophoresis, the protein concentration being about 4.5 mg. per milliliter; current intensity, 1.0 amp.; flow rates: top, 2 ml. per minute; bottom, 1 ml. per minute. The dialyzed and lyophilized top fraction is highly soluble, with an activity of 60–75 units per milligram. Electrophoretic and ultracentrifugal analysis (101) has shown the protein to be essentially homogeneous, except for a small slower-moving component, evident as a shoulder in the patterns presented in Fig. 18.

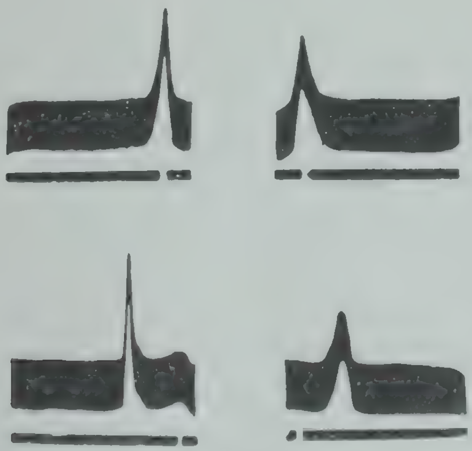

FIG. 18. Electrophoretic diagrams of elastase prepared by forced-flow electrophoresis (101). Top: pH 10; bottom: pH 5.

Peroxidase was fractionated according to the procedure of Theorell (102). Worthington horse-radish peroxidase, assaying approximately 60 units per milligram, is fractionated in 0.03 *M* phosphate buffer, pH 10.3, at a concentration of 10 mg. per milliliter, at a flow rate of about 0.5 ml. per minute. The top fraction is then crystallized by ammonium sulfate fractionation, yielding a product with an activity of about 320 units per milligram, P Z being 4800. In both instances, the smallest cell and window-type membrane were again utilized, accounting for the flow rates.

E. Selective Plasmapheresis

Forced-flow electrophoresis was found applicable also to the fractionation of whole blood, i.e. plasma and corpuscular elements included, provided that its clotting is prevented (103). The author is not aware of any other electrophoretic fractionation of whole blood recorded in the literature. Some of the results of this work are reported in Table XI, in all of the experiments a substantial separation of γ-globulin having been achieved. In aged blood bank samples, a certain amount of hemolysis was usually present; not so in fresh blood. The blood was generally diluted to one-half concentration with the buffer, and equilibrated against it by dialysis. The dilution was necessary, since in the course of the fractionation, the bottom effluent is concentrated to twice the inflowing concentration. No precipitation of either the corpuscular or the soluble components was observed at the membranes.

The possibility of isolating electrophoretically γ-globulin from whole blood has opened a way for a new technique of blood collection, for which the term "selective plasmapheresis" was suggested (103). Plasmapheresis (104) is a process in which the corpuscular elements of whole blood are centrifugally separated from the plasma and returned to the donor, only plasma being kept. This technique has recently been greatly improved with the development of the Cohn-ADL centrifuge (105), and some of the results were reported by Smolens *et al.* (106). In selective plasmapheresis, a fractionation of plasma proteins is superimposed on the separation of the corpuscular elements. In the experiments recorded in the last two lines of Table XI, a cannula was inserted into a leg vein of an anesthetized and heparinized dog and, at desired intervals, blood was drawn by means of a syringe provided with a four-way stopcock. In the syringe, the blood was then mixed with the buffer and injected into a reservoir, from which a continuous flow through the electrophoretic apparatus was maintained. The top effluent was retained and analyzed electrophoretically, while the bottom effluent was returned to the dog through the inserted cannula. In no instance was an untoward reaction observed in the animal.

Erythrocytes have electrophoretic mobilities which are largely un-

FRACTIONATION OF WHOLE BLOOD BY FORCED-FLOW ELECTROPHORESIS[a]

Blood	Buffer	pH	Amp.	Apparent concentration top effluent (%)					
				R.B.C.	Albumin	α_1	α_2	β	γ
Human, aged	0.4% Na citrate	7.4	1.2	0.2%	4	(		96[b]	)
	4.4% Dextrose								
	0.01 *M* Na barbital		1.5	0.2%	5	(		95[b]	)
Human, aged	0.4% Na citrate	7.6	.6	0.5%	4	0	9	18	69
	4.4% Dextrose		1.2	0.2%	0	0	0	8	92
	0.01 *M* Na barbital								
Human, aged	0.4% Na citrate	7.2	.6	5%	17	4	8	8	63
	4.4% Dextrose		.9	0.2%	11	6	0	9	74
	0.01 *M* Na barbital		1.2	0.1%	0	0	0	7	93
Human, fresh	0.4% Na citrate	7.1	.9	1%	21	3	5	9	62
	4.4% Dextrose								
			1.2	0.3%	13	0	0	16	71
			1.5	0%	0	0	0	10	90
Dog, selective plasmapheresis	0.4% Na citrate	7.2	1.2	2%	13	(		87[b]	)
	4.4% Dextrose		1.2	3%	30	(		70[b]	)
Dog, selective plasmapheresis	0.83% NaCl	7.1	1.5	0.5%	0	0	0	19	81
	0.03% KCl		2.0	0	0	0	0	21	79
	0.15% Na citrate								
	0.03% Heparin								

[a] Cell, 15 × 4.5 cm.; Whatman 54 center membrane; flow rate, 2.7 ml./min.; red blood cells (R.B.C.) determined by hematocrit, plasma by paper electrophoresis, fibrinogen not corrected for.

[b] Due to hemolysis the determination of globulin subfractions was not attempted.

affected by pH and strongly dependent upon salt concentration. These data are summarized in Chapter 10 of this book, by Brinton and Lauffer. In all instances, it was observed that the separation of the cellular components in forced-flow electrophoresis occurs at lower current intensities than the elimination of serum albumin. This is probably due to their larger size, i.e. tendency to sedimentation. In its present stage, selective plasmapheresis appears to be suitable for inducing experimental hypogammaglobulinemia, which may be of interest in the study of certain immunological problems.

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Addendum: Nonaqueous Solvents*

In his introduction to the present monograph, Tiselius pointed out the scarcity of electrophoretic data in nonaqueous solvents. A recent development in this field, while not related directly to electrophoresis, may prove to be of considerable importance for preparative purposes and it motivated this addendum.

The development is concerned with those effects of strong electric fields on dielectric particles which are caused by their dipole moment, permanent or induced, rather than by their electric charge. In an electric field, the dipoles will be more or less oriented, the particles having a finite separation of equal amounts of positive and negative charge. If the electric field is nonuniform, one end of the dipole will be in a weaker field than the other, and, as a result, the dipole will be attracted toward the place of greatest field intensity. In small particles, of molecular dimensions, Müller (1) and Lösche and Hultschig (2) have shown the effects to be small. The dipole moment of a spherical particle embedded in a dielectric medium being proportional to its volume, Debye (3) has considered the possibility of grading of polymers by molecular size.

The theory of the effects of nonuniform electric fields on macroscopic particles was further elaborated by Pohl (4, 5, 6), and put to experimental test (5, 7). *Dielectrophoresis* was defined as the motion of matter caused by polarization effects in a nonuniform electric field. It is related to phenomena observed by Leenov and Kolin (8) in inhomogeneous magnetic rather than electric fields, and termed *electromagnetophoresis*. In practice, it has to be differentiated from other competing phenomena, such as conduction, thermal convection, diffusion, and the effect caused by the charging of the particles on the electrodes, toward which they are attracted by dielectrophoresis, and subsequently repulsed by electrophoresis. Electrophoresis will, of course, always be present if the particles are charged, as was shown by van der Minne and Hermanie (9). Dielectrophoresis can be characterized by the following properties:

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* Added in proof.

1. The force of attraction exerted by the electric field is proportional to the cube of the particle radius, to the difference of its dielectric constant from that of the medium, and to the gradient of the *square* of the field strength.
2. This latter relationship contains the implication that the direction of the force is the same if the field be reversed in sign. In fact, the direction of the motion is independent of the field, i.e. direct or alternating current can be employed, polar particles moving always in the direction of greatest field intensity.
3. The effect is observable most readily in coarse suspensions (e.g. particle diameter $\geqslant 2\ \mu$).
4. It requires highly divergent fields. No motion should be observed in a uniform field.
5. The effect being rather small, it requires relatively high field strength, up to several thousand volts per centimeter.

The apparatus designed by Pohl and Schwar (5, 7) for the study of dielectrophoresis was a cylindrical tube, acting as one electrode, with a wire as the center electrode, the whole assembly reminiscent of the Clusius column. The suspension is fed from the top, and two outlets are provided on the bottom, one at the periphery of the tube, the other surrounding the center wire. With such cylindrical symmetry, the attractive force on the particles decreases with the cube of their distance from the center. The useful diameter of the cell is therefore limited, and significantly better results were obtained with a cell of internal diameter of 11 mm. than with a cell of 20 mm. in diameter. Experimental data reported have shown the usefulness of the cell for the separation of carbon black from suspensions in di-isopropyl ketone, and for the concentration of a suspension of polyvinyl chloride from a 1:1 v/v mixture of carbon tetrachloride and benzene. In both instances, the solvent being less polar than the suspensoids, the particles migrated toward the center wire. Reversing the polarity relationship, i.e. using a suspension of the nonpolar polyethylene in a 6:4 v/v mixture of acetone and nitrobenzene, the opposite effect was observed, though smaller in magnitude.

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Chapter 8

Applications of Moving Boundary Electrophoresis to Protein Systems

R. A. Brown and S. N. Timasheff

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I. INTRODUCTION

The method of Tiselius electrophoresis as applied to protein systems can yield a variety of information. The quantities measured experimentally are the areas under the boundaries formed during migration, the displacements of the boundaries, the shapes of the boundaries and their degree of spreading. From these measurements, it should be possible to obtain information on the zeta potential and charge of a protein molecule, the degree of homogeneity of a protein, the composition of a mixture of proteins, the effect of various reagents and treatments on the protein and the nature and degree of interaction of the proteins with themselves, with other proteins, and with ions or small molecules present in the solution. In previous chapters, a thorough theoretical treatment of these various topics has been presented. It is the purpose of this chapter to show by examples how some of these theories may be applied to actual systems and how an electrophoretic pattern may be properly interpreted. First we will treat the relation of mobility to the charge in the protein molecule and to its surrounding medium. Then, various types of heterogeneity will be treated, and finally the problems of interaction and modification of the molecule will be presented.

II. EFFECT OF BUFFERS ON ELECTROPHORESIS

A. Relation between Charge and Mobility

In general, there has been little activity in the use of Tiselius electrophoresis for the determination of zeta potentials and electric charges carried by proteins. One of the reasons for this apparent lack of interest is that the interpretation of electrophoretic mobilities in terms of charge is somewhat equivocal. There are some difficulties in the theoretical treatment, as shown by Overbeek in Chapter 1 of this monograph. Two excellent reviews of the theory of electrophoretic migration of colloids are available; one is in the book by Abramson *et al.* (1); the other is in a recent chapter by Overbeek (2). Only the results of the theory will be presented here, the reader being referred to Chapter 1 in this volume and the two reviews for a detailed treatment.

The relation between mobility, u, in cm.^{2}/volt-sec., and the zeta potential, ζ, as developed by Smoluchowski (3), Debye and Hückel (4), Hückel (5), and Henry (6) for a spherical particle of radius a is

$$u = \frac{D\zeta}{6\pi\eta} f(\kappa a)$$

$$f(\kappa a) = 1 + \frac{\kappa^2 a^2}{16} - \frac{5\kappa^3 a^2}{16} - \frac{5\kappa^3 a^3}{48} - \frac{\kappa^4 a^4}{96} + \frac{\kappa^5 a^5}{96} - \left(\frac{\kappa^4 a^4}{8} - \frac{\kappa^6 a^6}{96}\right) e^{\kappa a} \int_{\infty}^{\kappa a} \frac{e^{-t}}{t}\, dt \qquad (1)$$

where D is the dielectric constant, η is the viscosity of the liquid, $1/\kappa$ is the thickness of the Debye-Hückel ion atmosphere

$$1/\kappa = \left(\frac{DkT}{4\pi e^2 \Sigma u_i z_i^2}\right)^{1/2} \qquad (2)$$

k is Boltzmann's constant, T the absolute temperature, e the elementary charge, z_i the valence of the ions of species i, and n_i their number per cubic centimeter.

The function $f(\kappa a)$ approaches $\frac{3}{2}$ for spheres which are large when compared to the thickness of the double layer and 1 for very small spheres. Values of this function have been tabulated by Henry (6) [see also (1)] as a function of the ionic strength.

Henry's equation neglects the distortion of the double layer by relaxation effects. Overbeek (2) has shown that the correction for this effect is largest for intermediate values of κa, in the region of greatest interest for the protein chemist, i.e. for particles of dimensions between 10Å and 1μ and values of κ between 10^5 and $10^{7.5}$. A detailed treatment of this effect is available in Overbeek's articles.

The theory of ionic atmospheres (7) may be used to express the zeta potential in terms of the total charge, Q, on the protein. Taking into account the finite radius, a_i, (8) of the ions in the supporting electrolyte, one obtains

$$Q = D\zeta a \left[\frac{1 + \kappa(a + a_i)}{1 + \kappa a_i}\right] \tag{3}$$

For point charges this reduces to

$$Q = D\zeta a(1 + \kappa a) \tag{4}$$

Combination of equation (1) with equation (3) or (4) gives the relation between the charge on the protein and its electrophoretic mobility. In the application of the theory to actual protein systems several difficulties are encountered. The principal one is that the theory has not been extended to particles which are ellipsoidal in shape. Gorin [cf. reference (1)] has determined the relation between charge and zeta potential for cylindrical particles. This equation, which has been applied to ellipsoidal particles, is

$$u = \frac{Qf(\kappa a)(1 + \kappa a_i)}{6\pi\eta a[1 + \kappa(a + a_i)f(\kappa,a/b)C]} \tag{5}$$

The function $f(\kappa,a/b)$ has been tabulated by Gorin (1). It takes into account the axial ratio, a/b. C is a dimensional constant.

Little experimental work has been done on the relation of electrophoretic mobility to protein charge. Longsworth has determined the mobilities of ovalbumin over the pH range of 1.8–12.8 at 0.1 ionic strength (9) and pH 3.1–11.7 at 0.01 ionic strength (10). A comparison of his data at 0.1 ionic strength with titration data of Cannan *et al.* (11) is given in Fig. 1. In this figure, the number of equivalents of acid bound per gram of protein are plotted as ordinates on the left-hand side, the scale being shifted vertically so that the point of zero binding coincides with the electrophoretic isoelectric point of ovalbumin at 0.1 ionic strength, i.e.

pH 4.58. The mobilities are indicated by the circles, using the right-hand ordinate scale. An excellent parallelism between the two sets of data can be seen. When the charge was calculated from the mobility data, using equations (1) and (4), it was found that the electrophoretic charge values were only 60% of the titration charge over the entire pH range. Correction for the relaxation effects amounts to only 5% (2), while assumption of a cylindrical shape for the protein molecule increases the charge by 20% (1). Thus, a significant lack of quantitative agreement still exists.

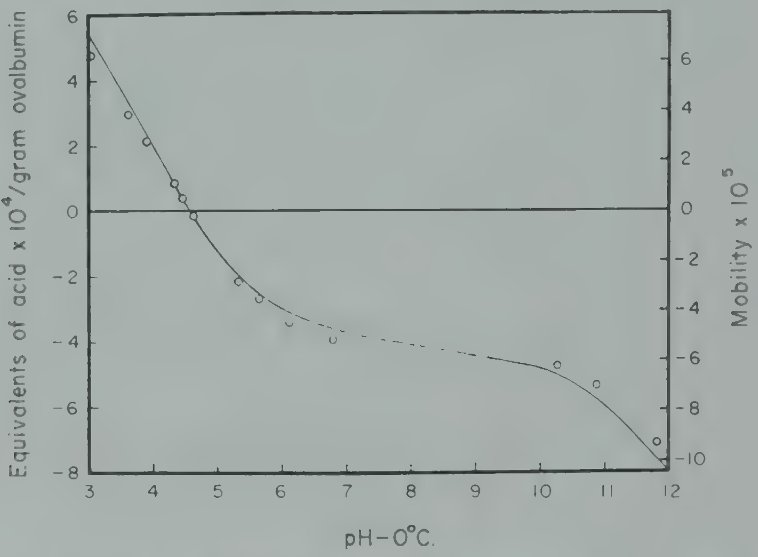


FIG. 1. pH-Mobility and titration curves of ovalbumin in 0.1 ionic strength (9). The mobilities are indicated by the circles, the curve represents the properly normalized titration curve.

A comparison has been made by Cannan and associates (12) of their titration data on β-lactoglobulin with electrophoretic data of Pedersen (13) at 0.2 ionic strength in the pH range between 3.2 and 8.9. In this case, the electrophoretic charge was found to be 85% of the titration charge. The better agreement in this case is explained by these authors as being due to the absence in β-lactoglobulin of the discrepancy between isoelectric and isoionic points found in ovalbumin.

A more recent study of this nature was carried out on trypsin by Duke and co-workers (14). Their results are presented in Fig. 2. For the sake of convenience, these authors chose pH 6.3 as the common point of reference for the two curves, so that the acid-binding capacity of the isoelectric protein is given by the sum of all the hydrogen and hydroxyl ions bound below pH 10.8, the isoelectric point. Again there is an excellent parallelism between the electrophoretic mobility and the acid-binding capacity of this protein. No comparison was made, however, of the charges calculated from

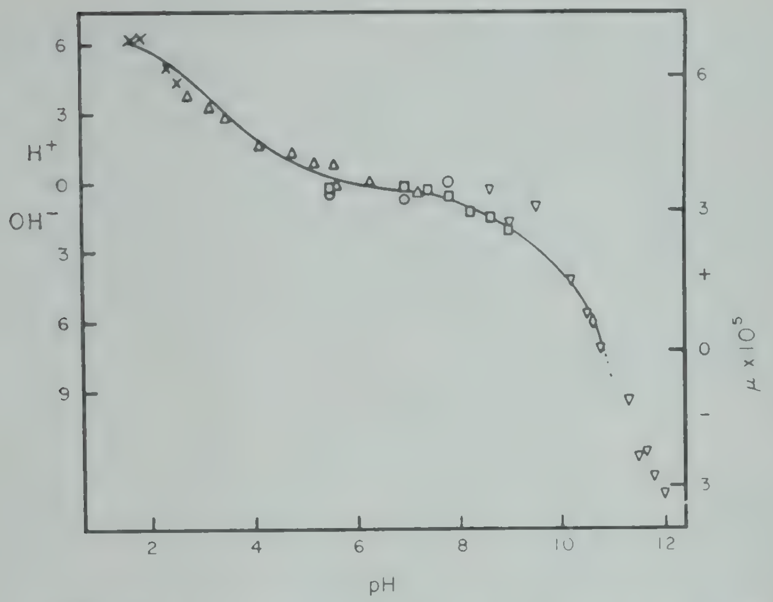


FIG. 2. Comparison of electrophoretic mobilities and dissociation curves of trypsin. Solid line: dissociation curve; points: electrophoretic mobilities in various buffers, ionic strength 0.13 (14). X, NaCl-HCl; △, NaAc-HCl; □, $CaCl_2$-barbiturate; ○, $MgCl_2$-barbiturate; ▽, $CaCl_2$-glycine; ◇, $CaCl_2$-NH_4Cl.

the two sets of data. The discrepancy between mobility and acid-binding capacity of some points measured in the presence of calcium ions may be attributed to the binding of these ions by the enzyme (14).

Velick (15) has used equation (5) to calculate the net charge of aldolase from the observed mobility in phosphate and acetate buffers of various ionic strengths. From these data he evaluated the number of phosphate ions bound per protein molecule and obtained good agreement with values calculated from dialysis equilibrium experiments. Comparison with titration data, however, resulted in a discrepancy that required eight times as many bound charges as found in dialysis equilibrium in order to reconcile the two sets of data. This discrepancy has been attributed by Velick to the probable effects of the nonavailability of some groups and to details of the molecular configuration.

The difference between titration and electrophoretic charge data has not been accounted for quantitatively. It would appear that the binding of counterions increases as the protein acquires a net charge. The zeta potential obtained in electrophoresis refers to the surface of shear. At high ionic strength, part of the counterions are present within this surface and by a steric effect diminish the electrophoretic charge, while they are not detected by other methods, such as titration experiments.

In recent years, the theory of titration curves has been developed considerably. A detailed treatment is given by Linderstrom-Lang and

Nielsen in Chapter 2. The relation (16, 17) normally used to relate pH to the dissociation properties of ionizable groups on proteins is

$$\mathrm{pH} - \log \frac{r_i}{n_i - r_i} = (\mathrm{p}K_{\mathrm{int.}})_i - 0.868wZ \tag{6}$$

where n_i is the number of ionizable groups of type i, r_i is the number of groups of type i dissociated at any pH, $(\mathrm{p}K_{\mathrm{int}})_i$ is the intrinsic dissociation constant of group i, and w takes into account the electrostatic interaction between the net charge Z of the protein at any pH and the dissociating hydrogen ions, and is equal to

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \tag{7}$$

where e is the protonic charge, D is the dielectric constant of the solvent, b is the radius of the spherical protein molecule, and a is the distance to which salt ions are excluded.

In the analysis of titration curves, the binding of ions other than hydrogen and hydroxyl is not taken into account. Furthermore, it is generally assumed that all ionizable groups are distributed evenly over the surface of the spherical molecule. Tanford (18, 19) has shown, recently, that the dissociation properties of ionizable groups on a protein molecule are quite sensitive to the location of the dissociable site with respect to the surface of the molecule, to the distribution of the ionizable sites with respect to one another, and to the ionic strength of the medium. Any change in these variables either in titration or electrophoretic experiments could result in significant variations of the net charge on the protein molecule.

Thus, while some data are available to show at least a semiquantitative relationship between charge and mobility, more work is necessary; especially desirable would be a series of studies correlating titration data with electrophoretic mobilities at different ionic strength and with ion binding taken into account.

B. Effect of Ionic Strength on Mobility

The Henry equation [equation (1)] has only a small explicit dependence on the ionic strength of the supporting electrolyte. $f(\kappa a)$ approaches $\frac{3}{2}$ at high salt concentrations and 1 at low concentrations. If one assumes, however, that the total charge on the protein is unaffected by the ionic strength, the zeta potential, and consequently the electrophoretic mobility, becomes strongly dependent on the ionic strength:

$$u = \frac{Kf(\kappa a)}{(\Gamma/2)^{1/2}} \tag{8}$$

where $\Gamma/2 = \frac{1}{2}\Sigma n_i Z_i^2$ is the ionic strength, and K is a constant.

Tiselius and Svensson (20) have measured the mobility of ovalbumin as a function of ionic strength at pH 7.10 and compared it with values calculated using the Henry equation. Their results indicate excellent agreement between the experimental and calculated values.

The effect of ionic strength on the isoelectric point has been studied for a number of proteins. Results presented in Fig. 3 for ovalbumin (20), carboxyhemoglobin (21), and aldolase (15) show a straight-line dependence of the isoelectric point on the square root of ionic strength. A similar linear relationship has been observed by Adair and Adair (22) in measurements of membrane potentials in the case of sheep hemoglobin. Such a dependence has been attributed by Tiselius and Svensson (20), at least partially, to the combination of buffer ions with the protein. Longsworth and Jacobsen (23) have shown that the data of Tiselius and Svensson (20) on ovalbumin may be represented, with an average deviation of 0.002 pH unit, by the relation

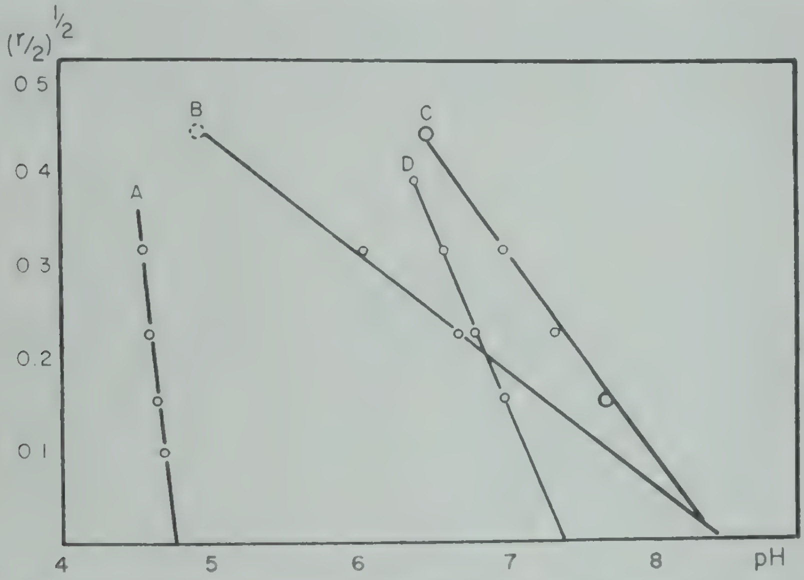


FIG. 3. Electrophoretic isoelectric points as a function of the square root of ionic strength. Curve *A*, ovalbumin in acetate; curve *B*, aldolase in phosphate; curve *C*, aldolase in acetate; curve *D*, horse carboxyhemoglobin in phosphate (15).

$$\mathrm{p}I = \mathrm{p}I_0 - \frac{a(\Gamma/2)}{1 + b(\Gamma/2)} \tag{9}$$

with $\mathrm{p}I_0 = 4.752$, $a = 5.0$, $b = 20$. This relation was obtained by combining a Langmuir adsorption isotherm with the assumption that the displacement of the isoelectric pH with increasing ionic strength is proportional to the change in net charge that accompanies the binding of buffer ions by the protein, i.e., $\mathrm{p}I = \mathrm{p}I_0 - k\Delta Z$.

A strong dependence of isoelectric point on ionic strength of human γ_1- and γ_2-globulins has been observed by Alberty (24), while similar data on bovine serum albumin and β-lactoglobulin has been obtained by Longsworth and Jacobsen (23). These authors point out the importance of the effect of bound salt ions on the ionization constants of ionizable groups on the protein. Anion binding is accompanied, at constant pH, by the binding of additional protons. Thus, if on partial substitution of the salt NaS for salt NaR at constant pH and ionic strength, s anions of S species are bound by each molecule of protein, displacing Δr of the R anions that are bound in solution of pure NaR and inducing the addition of Δh protons, the change in net charge is:

$$\Delta Z = -s + \Delta r + \Delta h \tag{10}$$

In a case where the amino acid composition is such that the protein is relatively weakly buffered in the isoelectric region, changes in bound charges are accompanied by a large pH shift, which is required for electrical compensation. In the case of aldolase, Velick (15) has found that the isoelectric point determined in 0.2 ionic strength phosphate buffer is 4 pH units less than the value calculated from amino acid composition.

C. The Moving Boundary Equation

In the electrophoretic analysis of protein mixtures for composition, it is found often that the apparent relative concentrations of components vary with the ionic strength of the buffer and the total protein concentration. These observations are the result of anomalies encountered in moving boundary electrophoresis. A thorough discussion of the theory of moving boundaries (25, 26) is given by Longsworth in Chapter 3 of this book. Using the notation of Longsworth the distribution of component j across boundary $\alpha\beta$ is given by

$$\frac{C_j^\alpha}{C_j^\beta} = \left(\frac{r_j}{\sigma^\beta} - V^{\alpha\beta}\right) \Big/ \left(\frac{r_j}{\sigma^\alpha} - V^{\alpha\beta}\right) \tag{11}$$

where r_j is the relative mobility, σ is the relative conductivity, $V^{\alpha\beta}$ is the volume swept out by boundary $\alpha\beta$ on the passage of one Faraday equivalent of electricity. The superscripts refer to the phase. Designating as component 1 the one which disappears across boundary $\alpha\beta$, it is found for component 2 that

$$\frac{C_2^\alpha}{C_2^\beta} = \left(\frac{r_2}{\sigma^\beta} - \frac{r_1}{\sigma^\beta}\right) \Big/ \left(\frac{r_2}{\sigma^\alpha} - \frac{r_1}{\sigma^\beta}\right) \tag{12a}$$

$$\frac{C_2^\alpha}{C_2^\beta} = 1 - \left[\frac{r_2}{r_2 - r_1} \cdot \frac{\sigma^\beta - \sigma^\alpha}{\sigma^\alpha}\right] + \cdots \tag{12b}$$

The consequences of these equations are discussed in detail by Longsworth (see Chapter 3). Several generalizations, however, can be made on the basis of the above equations. There is a concentration change across the boundary of any component with an electrophoretic mobility, and electrophoresis does not give in general the correct analysis of a mixture. Equation (12b) shows that the measured areas will deviate most seriously from the correct composition for two components with similar mobilities. This has been shown for hemoglobin, (27), β-lactoglobulin (28), and ovalbumin (29), among others. In order to analyze such systems, one may have to use calibration graphs of area distribution against known composition (27, 28, 30) or extrapolation of observed values to zero protein concentration at constant ionic strength (31) or to zero values of the ratio of protein concentration to ionic strength (32, 33).

Cann (29) has shown that in the case of ovalbumin the Dole theory (25) accounts adequately for the apparent composition of the protein as a function of concentration. (A detailed treatment of the heterogeneity of ovalbumin is given in Section III, A.) In this work the "true" electrophoretic distribution of components was determined by extrapolation of apparent distributions at finite concentrations to zero protein concentration at constant ionic strength. The deviations of the apparent from the extrapolated relative concentrations of component A_1 of ovalbumin when plotted against protein concentration were found to fall on straight lines, passing through the origin, as shown in Fig. 4. Theoretical deviations for two ovalbumin samples were calculated using the equations of Dole and are compared with experimental data in Fig. 4. The author concludes

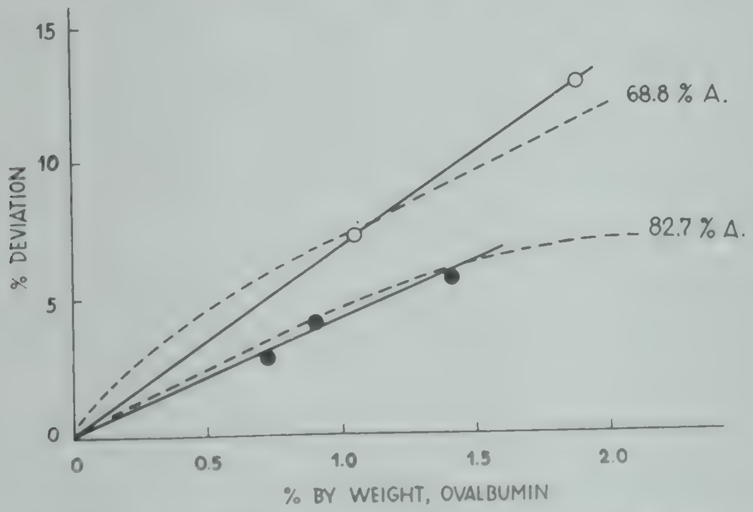


FIG. 4. Deviation of apparent from "true" electrophoretic distributions in ovalbumin (29): solid line, experimental deviations; broken curve, deviations calculated from the equation of Dole.

that the degree of agreement obtained is an adequate check of the Dole theory as applied to ordinary electrophoretic analyses of proteins.

Longsworth (31) has applied the theory of moving boundaries to systems consisting of a single protein, as well as to protein mixtures, and has shown that the net charge of ovalbumin may be calculated from the change in buffer concentration across the ϵ-boundary. A detailed analysis of this treatment is presented in Chapter 3 of this volume. Hoch (34) has examined the problem of boundary anomalies and concluded that relative concentrations in protein mixtures as deduced from apparent concentration changes at the boundaries are grossly erroneous when the difference in component mobilities is small. He further concluded that examination of a protein for homogeneity should be carried out at low concentrations, where the separation of boundaries is greatest. This can be carried out best with interference fringe optics, a procedure described in Chapter 4.

The foregoing analyses of moving boundary systems indicate that measured mobilities and areas should be extrapolated to zero protein concentrations. At zero concentration there will be no conductivity changes across the boundaries and the measured mobilities for all components will be correct. From equation (11) it can be seen that the ratio of concentrations across the boundary will become unity for all components which do not disappear across the boundary when the conductivities in the two phases are equal. This implies that measured areas which have been extrapolated to zero protein concentration will be correct.

One should add a word of caution, however. Equation (11) is based on the assumption that the relative mobilities are independent of phase. This appears to be a good assumption for strong electrolytes. It may be a rather poor one for weak electrolytes such as proteins. It has not been established yet that the analysis obtained by extrapolation to zero concentration is the correct one when dealing with weak electrolytes.

It has been shown that there are conductivity changes across the boundaries in electrophoresis. Since proteins in general bind hydrogen ions, there will be also pH gradients across the boundaries. The net result of these changes across the boundaries is either an increase or a decrease in the migration velocity of the protein across the boundaries and a consequent sharpening or broadening of the boundaries. These effects are treated in detail in Chapter 3.

Ideally the patterns in the two limbs should be mirror images. In practice the pH and conductivity changes across the boundaries cause deviations from enantiography. This phenomenon is common enough, so that the tendency is to overlook it. It should be realized, however, that marked deviations from enantiography are to be avoided since they are indicative of errors in the analysis. When the deviations are very marked it should be verified that the two limbs do approach mirror images at zero protein concentration.

The resolving power of the Tiselius method can be increased by taking advantage of the larger mobility differences and higher field strengths attainable at low ionic strength. Due to the presence of boundary-sharpening effects in the rising limb, best resolution into components is often obtained on that side. This can be used to advantage in the qualitative detection of components in a system.

Unfortunately, since the electrophoretic anomalies also increase at low ionic strength, in striving for increased resolution one is forced to compromise. Using 0.5% protein solutions, the results become equivocal below 0.05 ionic strength. The highly precise interference optics which are available now make it possible to work with lower protein concentrations. Using protein concentrations of the order of 0.1%, one may be able to take advantage of the improved resolution available at 0.01 ionic strength. Careful studies should be made, however, to see if the artifacts arising because of unstable boundaries and electroosmosis can be kept within tolerable limits under these conditions.

III. PROTEIN HETEROGENEITY

The most important use of electrophoresis is the analysis of heterogeneous mixtures. It is particularly useful in following fractionations of mixtures. A detailed discussion of the methods of obtaining relative concentrations of proteins from electrophoretic patterns is presented in Chapter 3. Cautions to be exercised, owing to the presence of boundary anomalies, have been described above and are treated in detail in Chapter 3. The application of electrophoresis to the analysis of complex mixtures of proteins and for following the progress of protein fractionations is discussed in Chapters 3, 4, and 7 of this book. Tiselius electrophoresis, however, is also a very powerful criterion of the homogeneity of single "pure" proteins. In recent years, with the use of this method, a number of proteins have been critically examined for homogeneity with the results showing a greater or smaller degree of heterogeneity in all. A few typical examples taken from these studies are discussed below. In most of the systems to be described below the differences between the discrete proteins are small and the resulting electrophoretic heterogeneity might not be immediately obvious. The electrophoretic resolution is often poor and might require a set of special conditions for detection. In some cases, resolution into components never occurs and the heterogeneity can be detected only from the spreading of the boundary.

A. Resolution into Boundaries

One of the most complete electrophoretic investigations of the homogeneity of a protein has been carried out on ovalbumin. As a result, a thorough understanding of that system is available now. The electrophoretic heterogeneity of ovalbumin has been investigated by a number of workers (29, 35–39). Longsworth (35) first pointed out that crystalline ovalbumin resolved into two electrophoretic components. A similar observation was made by Tiselius and Eriksson-Quensel (36). A detailed study of this problem (37, 38) revealed the presence of three components, designated as A_1, A_2, and A_3 in decreasing order of mobility on the alkaline side of the isoelectric point. These were found to vary in relative concentration between preparations. MacPherson and associates (38) further demonstrated that prolonged storage resulted in the disappearance of component A_1, accompanied by a large increase in the amount of A_2 present in the system. Perlmann (40–42) carried out a detailed study of the difference in components of ovalbumin and of the mechanism of their interconversion. She found that A_1, A_2, and A_3 differ primarily in their phosphorus content and have 2, 1, and 0 phosphate residues per mole, respectively. The mobilities of the three components at pH 6.8 in phosphate buffer of 0.1 ionic strength and their isoelectric points are given in Table I. Such differences in mobility and isoelectric pH have been shown to correspond to a change in the net charge of -2 per phosphorus atom (42). Dephosphorylation of A_1 to A_2 was carried out with prostate phosphatase at pH 5.35 or intestinal phosphatase at pH 9, with the accompanying electrophoretic changes shown in Fig. 5. Conversion of A_2 to A_3 could be carried out only with intestinal phosphatase at pH 5.3. This difference in splitting may

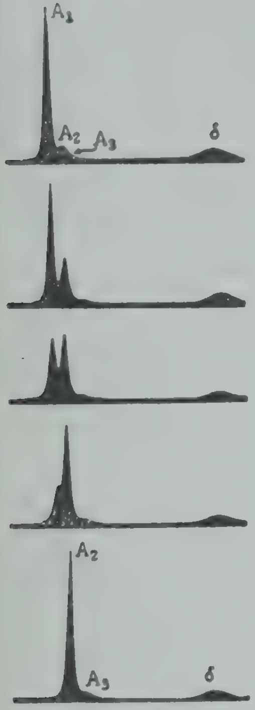
TABLE I

ELECTROPHORETIC PROPERTIES OF OVALBUMINS AND PLAKALBUMINS[a]

Protein	A_1	A_2	A_3	P_1	P_2	P_3
Crystal form	Needles	Needles	Needles	Plates	Plates	Plates
Atoms phosphorus per mole of protein	2	1	0	2	1	0
$u \times 10^5$ cm.^{2}/sec. v.[b]	−6.1	−5.2	−4.5	−5.5	−4.6	−3.8
Isoelectric point at 0.1 ionic strength	4.58	4.65	4.74	4.72	4.8	

[a] According to Perlmann (42).

[b] Electrophoresis in pH 6.8 phosphate buffer of 0.1 ionic strength.



Time in hours	Electrophoretic composition	Atoms phosphorus per mole protein	
		Computed	Observed
0	85% A_1 14% A_2 Trace A_3	1.8_4	1.8_2
½	58% A_1 40% A_2 Trace A_3	1.5_6	1.4_8
¾	47% A_1 49% A_2 4% A_3	1.4_3	1.3_5
1¾	36% A_1 58% A_2 6% A_3	1.3_0	1.2_0
7	94% A_2 6% A_3	0.9_4	0.9_7

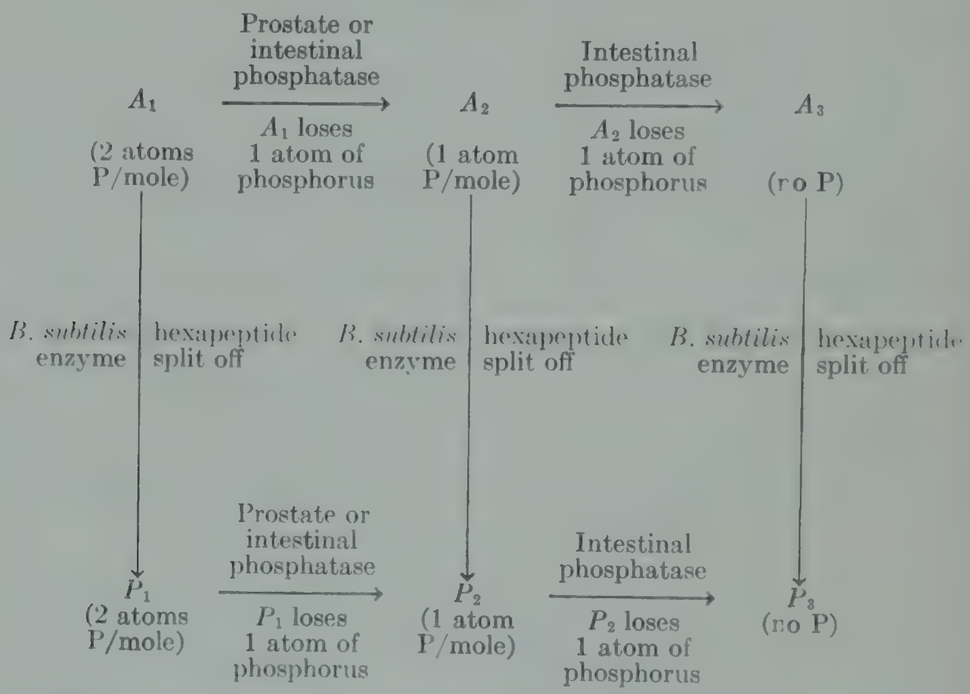
FIG. 5. Changes in electrophoretic patterns as a result of dephosphorylation of ovalbumin with prostate phosphatase as a function of time (42).

reflect a difference in the chemical binding of the two phosphate residues.

Linderstrøm-Lang and Ottesen (43, 44) have found that another enzymatic modification of ovalbumin could be isolated from protein solutions that had been stored for a long time and were found to be infected by bacteria or molds. The crystals formed by this protein were in the form of plates rather than needles. As a result, the authors called this protein plakalbumin. It was shown by these authors that ovalbumin incubated with an enzyme preparation from *Bacillus subtilis* formed plakalbumin in yields up to 90%.

Perlmann investigated the electrophoretic properties of plakalbumin and compared them to those of ovalbumin (45, 46). The mobility of plakalbumin was found to be more positive between pH 3 and 7 than that of ovalbumin and its isoelectric point to be 0.14 pH unit higher. The patterns obtained with plakalbumin showed two components P_1 and P_2 corresponding to A_1 and A_2 of ovalbumin, the phosphorus content of plakalbumin being identical with that of ovalbumin (40, 41, 44). Treatment of plakalbumin with prostate and intestinal phosphatase resulted in changes paralleling those observed in ovalbumin (41, 42), component P_2 being obtained either from plakalbumin by treatment with prostate phosphatase or from A_2 by treatment with *Bacillus subtilis* enzyme. Ege-Larsen and associates (47) found that the action of *B. subtilis* consisted in the splitting off of peptides, while Ottesen and co-workers (48, 49) identified the peptide as the hexapeptide alanylglycylvalylaspartylalanylalanine, split off the C-terminal end of ovalbumin. The over-all ovalbumin-plakalbumin transformation is summarized in Table I, and in reaction scheme I.

SCHEME I


Prostate or intestinal phosphatase
Intestinal phosphatase
A_1
A_2
A_3
A_1 loses 1 atom of phosphorus
A_2 loses 1 atom of phosphorus
(2 atoms P/mole)
(1 atom P/mole)
(no P)
B. subtilis enzyme
hexapeptide split off
B. subtilis enzyme
hexapeptide split off
B. subtilis enzyme
hexapeptide split off
Prostate or intestinal phosphatase
Intestinal phosphatase
P_1
(2 atoms P/mole)
P_1 loses 1 atom of phosphorus
P_2
(1 atom P/mole)
P_2 loses 1 atom of phosphorus
P_3
(no P)

These studies can serve as an excellent example of the combination of electrophoretic and chemical techniques in the unraveling of a complicated system. One should point out that in this case the net charge differences between individual proteins were of the order of one to two electronic charges, yet excellent electrophoretic resolution could be obtained.

A similar case of electrophoretic heterogeneity is found in ovomucoid (37, 50–53). In this case, the heterogeneity is more difficult to detect. Although at normal ionic strengths no serious heterogeneity was evident, Frédéricq and Deutsch (51) found that, if electrophoretic analyses were carried out at 0.01 ionic strength at pH 4.5, five distinct electrophoretic peaks could be observed on the ascending side. This is a good demonstration of the higher degree of resolution that can be obtained in the ascending limb at low ionic strength, which has been pointed out above. Electrophoretic patterns of this protein are given in Chapter 7. Bier and co-workers (52, 53) proved that the appearance of five peaks in electrophores was due to the presence of discrete proteins by fractionation of ovomucoid by electrophoresis-convection (see Chapter 7). They succeeded in isolating O_1 in pure form and also obtained fractions enriched with respect to O_2 and O_3. The isoelectric points were found to be O_1, pH 4.41; O_2, pH 4.28; O_3, pH 4.17; O_4, pH 4.01; O_5, pH 3.83. Mobility measurements on the initial protein and various fractions indicated the absence of interactions among the various components of ovomucoid. Since this protein is known to act as a trypsin inhibitor (54), these authors tested the various fractions for this activity. Their results showed equal antitryptic activity in all the major fractions, showing ovomucoid to be a striking example of a set of electrophoretically discrete proteins originating from the same source and possessing the same biological properties.

Another electrophoretically heterogeneous, biologically active protein is the enzyme trypsin. Its heterogeneity is found, however, only under a very special set of conditions. This protein normally has electrophoretic properties typical for a "homogeneous" protein. Bier and Nord (55, 56) have shown that if the electrophoretic analyses were performed in the presence of calcium, manganese, or cadmium ions, a second minor, more slowly migrating, component appeared. These ions are known to be bound by trypsin and to stabilize it against self-digestion (57, 58). When the electrophoretic experiments were carried out in the presence of other divalent ions which have no effect on the biological activity of trypsin, no resolution into components occurred. Timasheff and associates (59) isolated the rapidly migrating component in the separation cell of the Tiselius electrophoresis apparatus and examined it for electrophoretic heterogeneity and enzymatic activity. As shown in Fig. 6, the isolated material migrates as a single component. Furthermore, no difference in enzymatic activity was found between the original protein and the pure, rapidly migrating component. It should be noted that in this case the presence of discrete closely related proteins in a single crystalline system could be demonstrated only under very special conditions, involving the specific binding of ions. Thus, one can see that great caution should be exerted in drawing conclusions on the homogeneity of a protein from electrophoretic measurements even if no splitting into components occurs over a wide pH range.

Cunningham (60) found that when trypsin is inhibited by reacting with diisopropylfluoro phosphate (DFP), the resulting material displays the electrophoretic heterogeneity shown in Fig. 6a over the pH range of 7.9 to 11.3, whether calcium ions are present or not. In the presence of calcium ions, the mobility of the major component increases, indicating binding of the divalent ions by the inactivated enzyme, as is the case with the active trypsin (61). Ram and co-workers (62) found that acetyl trypsin also displays the heterogeneity observed with normal trypsin, but the

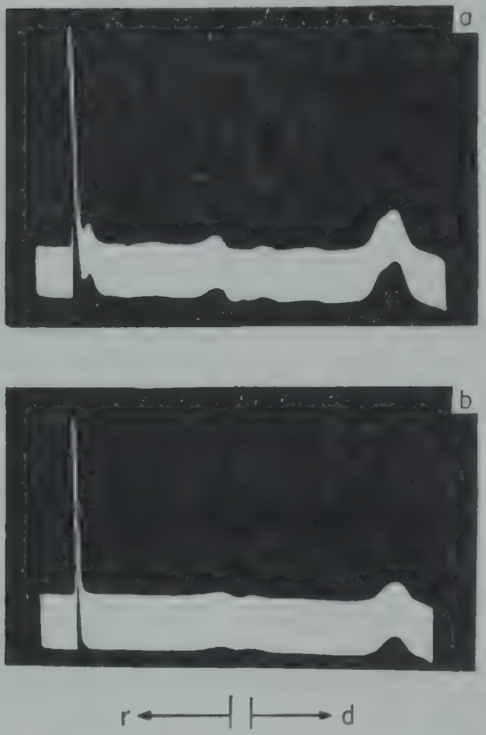


FIG. 6. Electrophoretic diagrams of trypsin preparations (59): a. Original whole trypsin. b. Rapid component after isolation in electrophoresis separation cell. Ca acetate buffer; Γ/2 = 0.1; pH = 4.7; 1800 seconds at 12 volts per centimeter.

presence of specific ions is not necessary for this. It is enzymatically active, and stable to self-digestion. It has an isoelectric point of 3.8, whereas that of normal trypsin is 10.8. It is interesting to note that in both cases of chemical modification, the electrophoretic heterogeneity becomes more easily detectable, while enzymatic activity is destroyed in one case only.

One of the most interesting recent applications of moving boundary electrophoresis has been the studies of various hemoglobins. This topic is discussed in Chapter 9. In the case of normal and sickle-cell hemoglobin, Ingram (63) has found that the two proteins differ by a single amino acid involving only one charge. And yet excellent electrophoretic resolution is obtained, showing once again the extreme sensitivity of the method of Tiselius electrophoresis for detecting very small charge differences between two proteins.

Shooter and associates (64, 65) have carried out a study on mixtures of various hemoglobins, aimed at showing the importance of the proper selection of buffer type and ionic strength for maximal resolution. They found that if a phosphate buffer of pH 6.40 and 0.04 ionic strength is used, good resolution is obtained between components in the ascending channel. Typical patterns comparing the resolution of hemoglobins A and S in various buffers are shown in Fig. 7. The area distribution under the peaks is far from the true composition of the system, but for component identification purposes such a degree of resolution may be often desirable. The advantage of using a buffer in which ascending boundaries are artificially

BUFFER	pH	I	F (volts/cm.)	MIGRATION TIME (HOURS)	HEMOGLOBIN CONC (%).	CELL OPTICAL DEPTH (mm.)
CACODYLATE CHLORIDE[1]	6.48	0.10	3.6	15	1.0	25
CACODYLATE CHLORIDE[1]	6.48	0.10	3.6	15	1.0	10
BARBITURATE[2]	8.76	0.06	7.4	6	1.0	10
PHOSPHATE[3]	6.40	0.04	7.4	5	1.86	10

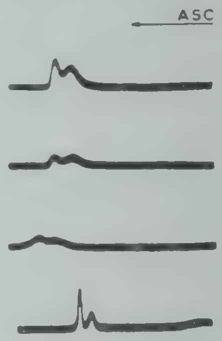


FIG. 7. Comparison of the analysis of hemoglobins A and S in buffers employed by different authors (65) [1. L. Pauling, H. A. Itano, S. J. Singer, and I. C. Wells, *Science*, **110**, 543 (1949); 2. D. B. Morrison, R. P. T. Rudnicki, and L. W. Diggs, *Federation Proc.* **13**, 267 (1954); 3. E. M. Shooter and E. R. Skinner, *Biochem. J.*, **60**, xxviii (1955).]

sharpened is further demonstrated by the complete resolution of a number of hemoglobin pairs and combinations of three in the 0.04 ionic strength phosphate buffer.

Hoch (66), in prolonged electrophoretic analysis of pepsin has shown this protein to be a mixture of four components. In this work electrophoresis was carried out with counter-compensation, and resolution into components could be obtained only after 30 cm. of migration. During short-time electrophoresis, this protein appeared to be fairly homogeneous (96% migrated as a single component at pH 3.9 and 5.9), while prolonged runs in pH 5.9 phosphate buffer of 0.925 ionic strength resulted in the resolution of the four components. The pattern was analyzed using the equation of Hoch-Ligeti and Hoch (67). It was found that, although the apparent proportion of the major component was 66%, its actual concentration is only 43%. The relative difference in migration velocities of the two main components was 1.5%. Thus, electrophoretic resolution was achieved even though the components had almost identical mobilities. Such systems can be analyzed quantitatively, however, only with the very careful application of the moving boundary theory; otherwise very large errors result in the composition analysis.

Another interesting application to a biological system is found in the work of Singer and co-workers (68), who showed the presence of two different strains of virus (tobacco mosaic virus) in electrophoretic experiments. The new strain was both detected and isolated electrophoretically.

Beaven *et al.* (69) have studied electrophoretically the difference between human fetal and adult hemoglobins, developing a method of analysis of each in the presence of the other.

A protein showing a very complex pattern of heterogeneity is β-lactoglobulin. This protein, which is the main component of milk whey, has been found by many workers to be heterogeneous below pH 5.2 (23, 28, 70–75) (its isoelectric point), while disagreement exists on its heterogeneity above this pH. The electrophoretic heterogeneity of β-lactoglobulin below pH 5.2 has been attributed by Longsworth and Jacobsen (23) to the presence of interactions and will be treated later. In the pH region between 5.3 and 6.0, Li (70) and McMeekin and co-workers (71, 72) failed to observe heterogeneity in short-duration electrophoresis experiments. Smithies (74) however, using a variety of techniques including electrophoresis, showed this protein to be heterogeneous in the same pH region. Following the discovery of Aschaffenburg and Drewry (76) that β-lactoglobulin consists of two genetically different proteins, Timasheff and Townend (28) demonstrated that its electrophoretic heterogeneity between pH 5.3 and 6.0 can be accounted for by the presence of these two proteins. In this work, prolonged electrophoretic analyses were carried out on the two individual proteins, obtained from the milk of individual cows (76), as well as on mixtures of the two proteins which Aschaffenburg and Drewry (77) designated as β-lactoglobulins *A* and *B*. The areas under the individual boundaries were found not to correspond to the correct known distribution of the two components. Therefore, a calibration graph was made using mixtures of known composition. In this way it was shown that normal β-lactoglobulin obtained from pooled milk consists of 60% *A* and 40% *B*, while the area distribution under the electrophoretic pattern gives an apparent composition of 76% *A*. This large difference between the actual composition and the area obtained on synthetic mixtures is the result of the superposition of boundary anomalies on a slight skewness of the pattern of pure β-lactoglobulin *B*. The mobilities of the two components are −0.63 (*A*) and −0.12 (*B*) at pH 5.3, and −1.67 (*A*) and −1.46 (*B*) at pH 5.6 in acetate buffers of 0.1 ionic strength. Their isoelectric points are 5.09 (*A*) and 5.23 (*B*) (78). Such differences in mobility and isoelectric points should correspond to about two charges. The electrophoretic heterogeneity of this system is complicated by the presence of protein association below pH 5.2. In the pH region above 5.2 it was shown that no association takes place (79).

In some cases electrophoretic heterogeneity can be detected only by the appearance of a shoulder on the main peak with no actual separation of components. Such is the case in insulin, which was studied by Timasheff and co-workers (80, 81). In order to prove that the heterogeneity in this system is due to the presence of different proteins, insulin was fractionated by electrophoresis-convection (see Chapter 7). An attempt to correlate this heterogeneity with the observation of Harfenist and Craig (82) that insulin contains two components, *A* and *B*, differing by one carboxylic charge per 12,000 molecular weight, resulted in the finding that the Craig component *A* displays an electrophoretic heterogeneity equal to that of whole insulin, but a mean mobility 0.12 mobility units more positive than the whole insulin. Since apparently the electrophoretic heterogeneity of insulin cannot be accounted for in terms of chemical differences, it would seem that it is probably related to some differences in molecular folding and hydrogen-bonding between individual molecules (83). The carboxylic charge difference seems to play only a secondary role.

B. Boundary Spreading

In the previous discussion the components of the mixture to be analyzed could be considered as discrete. There are actually a considerable number of protein preparations which are heterogeneous but which travel as a single peak in the electrophoresis apparatus. The heterogeneity of these preparations is demonstrated best by the techniques of reversible electrophoretic boundary spreading which have been developed by Alberty and others (84–86). A detailed description of this phenomenon is given in Chapter 3. In the application of this technique, correction for diffusion is usually carried out by extrapolating the function $g(u)$ [see Chapter 3, equation (45)] to zero reciprocal time. Brown and Cann (85) however, have shown that the mobility may be expressed also in terms of the moments of the refractive index gradient, X^n.

$$g(u) = \frac{1}{\beta\sqrt{2\pi}} e^{-u^2/2\beta^2} \left\{1 + \sum_{j=3}^{\infty} \frac{C_j}{j!} (-i)^j \alpha^j H_j\left(\frac{iu\sigma}{Et_E{}^2\beta^2\alpha}\right)\right\} \tag{13}$$

$$\beta^2 = \frac{\sigma^2 - \sigma_0{}^2 - 2Dt_E}{E^2 t_E{}^2} \tag{a}$$

$$\alpha = \sqrt{1 - 2(\sigma^2/\beta E t_E)^2} \tag{b}$$

The $H_j(\xi)$ are the Hermite polynomials and the C_j are the coefficients of the Gram-Charlier series (87). σ_0 is the standard deviation of the gradient curve at the moment the field is applied, σ is the standard deviation after electrophoresis for t_E seconds, E is the electric field. The other terms are the same as used by Longsworth (see Chapter 3, page 134). Since it is very difficult to obtain accurately moments of the refractive index gradient higher than the second, the usefulness of equation (13) is in general limited to the determination of the second moment of the mobility distribution. A number of proteins have been examined in reversible boundary-spreading experiments and their heterogeneity constants have been determined. The significance of these analyses is discussed in Chapter 3 and the values of *h* for a number of proteins have been tabulated by Alberty (88).

The final unequivocal proof of protein heterogeneity is best provided by the isolation of the individual components. In the case where discrete components are present, fractionation can be carried out by a number of techniques. In the case of a heterogeneous system with a continuous distribution of mobilities, Cann *et al.* (89) have shown that fractionation may be carried out very successfully by means of electrophoresis-convection (see also Chapter 7). These authors fractionated bovine γ-globulin into eight fractions differing from one another by at least two electrophoretic properties, such as isoelectric point, mean mobility at pH 8.7 and mobility distribution at the mean isoelectric point. These fractions constitute a mean mobility spectrum of from $-1.25\ \mu$ to $-2.25\ \mu$ at pH 8.7 and range in isoelectric points from 7.31 to 5.74. The isoelectric point of the starting γ-globulin was 6.75 and its mobility $-1.73\ \mu$. A considerable variation in $\Delta u/\Delta \mathrm{pH}$ was found among the fractions, reflecting probable chemical and structural differences. The normalized mobility distributions of six such fractions at pH 6.5 are plotted in Fig. 8. The starting material for the preparation of fractions G and H was non-Gaussian with the result that fraction G (top) exhibited a non-Gaussian mobility distribution at its

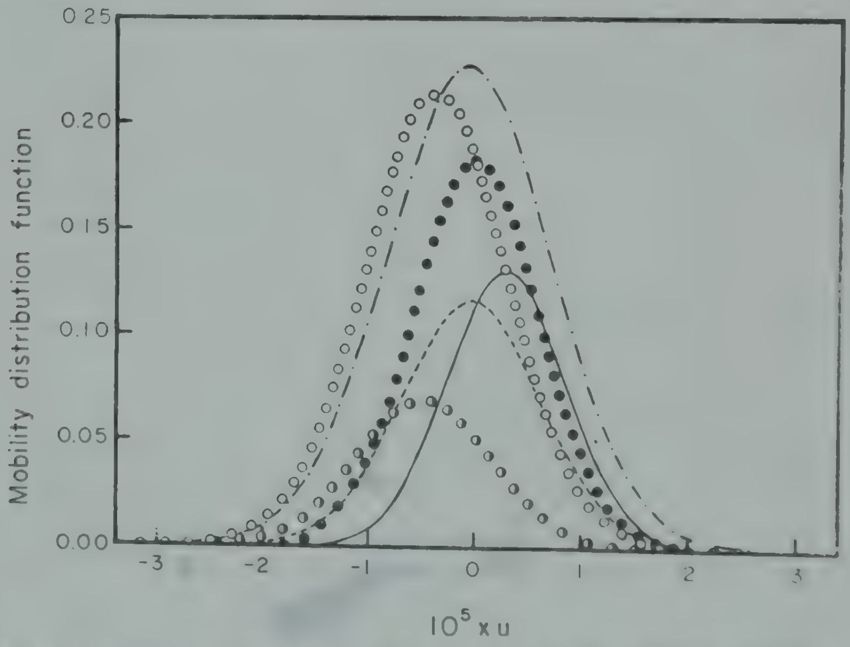


FIG. 8. Mobility distribution functions at pH 6.5 of six unique fractions of bovine γ-globulin: = fraction C (pI, 6.47); ○ = fraction D (pI, 6.01); = fraction E (pI, 7.31); ● = fraction F (pI, 6.51); ◑ = fraction G (pI, 5.74); = fraction H (pI, 6.41) (89).

mean isoelectric point. This is in agreement with the theory of transport in electrophoresis-convection which predicts that the fractionation of a protein possessing a Gaussian mobility distribution will result in a top fraction which also possesses a Gaussian mobility distribution, having the same second moment as that of the original protein, while a starting material with a non-Gaussian mobility distribution will result in a non-Gaussian top fraction.

C. *Microheterogeneity*

In the previous two sections, a description was given of various degrees of protein microheterogeneity which can be detected by electrophoresis. In some cases, such as ovomucoid, it is possible to obtain almost complete electrophoretic resolution of the component proteins, even when the differences in physical properties are quite small. In such cases, it is often desirable to take advantage of the increased degree of resolution which can be afforded by boundary sharpening on the ascending side, even if quantitative analysis of composition has to be sacrificed. In the case of hemoglobin the difference between types A and S is a single charge, in ovalbumin the difference between the various components amount to one or two charges. One is tempted to ask what is the nature of the more subtle heterogeneity which is found in other proteins. In the case of γ-globulin, different electrophoretic fractions have been found to have different biological activities (see Chapter 7). In most cases, however, there has been no evidence associating biological heterogeneity with boundary spreading in the electrophoresis apparatus. An excellent review of the general problem of protein microheterogeneity has been written by Colvin *et al.* (90).

In some cases, the microheterogeneity has been correlated with definite chemical differences, such as in ovalbumin and hemoglobin. In the case of trypsin, heterogeneity seems to depend on the differential binding of some divalent ions by the two species. Insulin presents a case in which a known chemical difference between components (one charge per molecule) seems to be superseded by differences in secondary and tertiary structure, as evidenced by possible changes in hydrogen-bond formation shown by Frédéricq (83) and Laskowski *et al.* (91). In general, however, a continuous mobility distribution is found in a number of crystalline highly purified proteins. Part of this could be due to differences in chemical structure. For example, in serum albumin, it is known that there are two components, one of which has a free —SH group while the other one does not. It is possible that there are random variations in the spatial configuration of the nonessential parts of the protein. Such variations would affect the ionization constants of the amino acids present in the protein as shown by Tanford (18, 19). Structural variations could be of various natures.

They could be the result of differences in folding in various individual molecules, or possibly minor variations in the amino acid sequence or even composition. Furthermore, one could conceive of the existence of dynamic configurational changes in protein molecules, very slow relative to the length of an electrophoresis experiment, but sufficient to lead to the observed boundary spreading. One should also not exclude small differences between individual molecules in the effects of pH, ionic strength, and type of ions present in the system as well as the effect of the application of the electric field. Another possibility is afforded by differences in the kind and amounts of carbohydrate or hydrocarbon materials which may be tightly bound to the protein molecules. These could either contribute to the charge, or affect the folding of the molecule leading to small changes in its hydrodynamic or electrostatic properties. All these differences could lead to slight differences in electrophoretic mobilities between individual molecules. These small differences could also be exerted through variations in ion binding and molecular unfolding which might have considerable effects on the ionization of vicinal groups (18, 19). With the exception of minor differences in amino acid sequence or composition in a small number of molecules, all of these variations are in spatial configuration. It is difficult to conceive of another explanation for the microheterogeneity of proteins in view of the apparent homogeneity found by the organic chemists in all the proteins that they have studied. It is true that minor variations in a small number of molecules could go undetected by the present techniques of structural protein organic chemistry.

IV. Interactions in Protein Systems

In addition to giving information on the ionization and degree of homogeneity of a protein, Tiselius electrophoresis can yield information also on various interactions which occur in the system. The following types will be considered here: protein-ion interaction, interaction of different proteins with each other, polymerization of a protein, and isomerization of proteins. No attempt will be made to give a complete coverage of these topics, but it will be attempted to show by examples how such systems can be analyzed properly.

A. Protein-Small Ion Interactions

The binding of small ions by proteins has been studied in several cases by applying the theory of weak electrolytes in moving boundary systems. It is discussed in detail in Chapter 3, Sections IV and V. A typical example of such a study is the work of Alberty and Marvin (92) on the binding of chloride ions by bovine serum albumin, described in Chapter 3, Section V, C. Smith and Briggs (93) adapted the theory of weak electrolytes in a somewhat different manner to a study of the binding of methyl orange by bovine serum albumin (BSA). In experiments using boundary system I of Chapter 3, Section V, A, it was found that the constituent mobility of the protein is a linear function of the amount of dye bound. In order to determine the amount of dye bound by the protein, using electrophoretic data alone, a second set of experiments using boundary system II of Chapter 3, Section V, A was carried out.

Figure 9 shows the boundary system to be expected in the descending limb. The boundary $\alpha\beta$ represents the hypothetical dye boundary. Its velocity may be determined in a separate experiment if one assumes that the protein has a negligible effect upon the mobility of the free dye. The dye disappears across the $\beta\gamma$ boundary, and its constituent mobility may be calculated from that boundary. The $\gamma\delta$ boundary gives the apparent mobility μ_p' of the protein, which differs from the true mobility of the protein, μ_p, by the factor $\kappa^\alpha/\kappa^\gamma$. All the free dye present in the original solution has passed the boundary $\alpha\beta$. Therefore, the free dye now present in β must have been bound by the protein in γ. We may set the amount

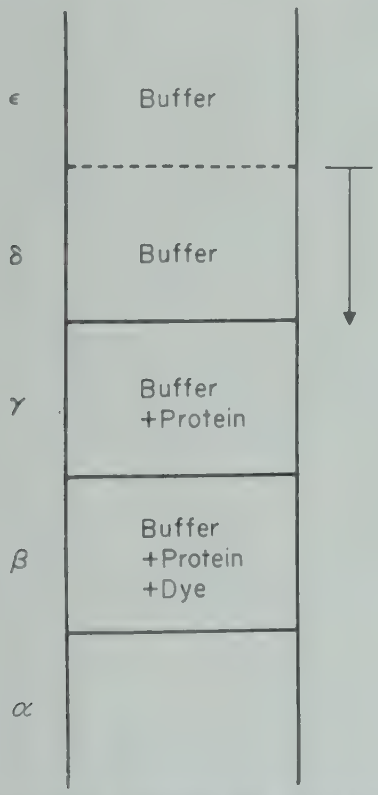


FIG. 9. Diagram of boundaries in descending limb after electrophoresis of albumin-methyl orange mixture (93).

of dye originally bound by the protein in γ equal to the free dye in β. If one assumes a unit cross section for the cell, volume can be replaced by mobility differences and we get

$$[A] = [A_0] \frac{\bar{\mu}_A - \bar{\mu}_p{}'}{[P_0]/[P]\gamma(\mu_A - \bar{\mu}_A) + \bar{\mu}_A + \bar{\mu}_P{}'} \tag{14}$$

Where $[A]$ is the molar concentration of free dye; $[A_0]$ is the total molar concentration of the dye, and $[P_0]$ is the molar concentration of the protein.

$[P]^\gamma/[P_0]$ may be approximated from the areas, I, of the descending pattern and the area due to the total dye, I_{A_0}.

$$\frac{[P]^\gamma}{[P_0]} = \frac{I_{\gamma\delta}\left(1 + \dfrac{I_{\gamma\epsilon}}{I_{\gamma\delta} + I_{\beta\gamma}}\right)}{I_{\gamma\delta} + I_{\beta\gamma} + I_{\delta\epsilon} - I_{A_0}} \tag{15}$$

The latter can be obtained from the refractive index increment of the dye. Equation (14) enables one to calculate the amount of free dye in a protein solution from parameters measured in the electrophoresis apparatus. When the electrophoretic data were plotted by the method of Klotz *et al.* (93a), values for n (maximum number of binding sites in the protein) and K (dissociation constant) were found to be 22.0 and 2.83×10^{-4}, respectively. These are in fair agreement with dialysis experiments which have given 22.6 for n and 3.12×10^{-4} for K.

A recent electrophoretic study of the interaction of ions with serum proteins has been carried out by Schilling (94). The difference in electrophoretic mobilities in the presence and absence of cadmium, lead, silver, zinc, and sulfosalicylate ions was measured. A detailed study of the interaction of Cd^{++} ions with BSA was carried out. If it is assumed that ions are bound by n equal sites, where they are competing with hydrogen ions, the observed electrophoretic mobility change (Δu) is determined by

$$\Delta u = \frac{2\nu + \nu_H - \nu_{H_0}}{f} \tag{16}$$

where ν is the number of sites occupied by cadmium, ν_H those occupied by hydrogen ions, f is the number of charges corresponding to a unit mobility change, ν_{H_0} is the number of sites occupied by hydrogen at zero cadmium concentration. The mass action law then gives:

$$\frac{1}{f\Delta u} = a \frac{1}{[Cd^{++}]} + b$$

$$a = \frac{(1 + K_H[H^+])^2}{nK(2 + K_H[H^+])};\ b = \frac{1 + K_H[H^+]}{n(2 + K_H[H^+])} \tag{17}$$

where K and K_H are equilibrium constants for the binding of cadmium and hydrogen ions, respectively.

A plot of $1/\Delta u$ against $1/\mathrm{Cd}^{++}$ resulted in a straight line yielding a binding constant in agreement with that found for attachment to histidine residues.

In other studies on the binding of ions to proteins, Volkin (95) used the shift in the pH-mobility curve of insulin in the presence of thiocyanate ions, to show the binding of these ions to basic groups on that protein. Nord and Bier (56) have used similar information to show the binding of calcium ions to trypsin. The combination of detergents with proteins is another special case of small-ion binding. It has been investigated by means of electrophoresis on a number of systems. In studies on anionic detergents, Lundgren and associates (96) studied the interaction of ovalbumin with alkylbenzene sulfonates; Putnam and Neurath (97, 98) studied the interaction of serum albumin with sodium dodecyl sulfate; Ballou and co-workers (99) investigated the effect of lower fatty acid salts on the mobility of human serum albumin. Timasheff and Nord (100) investigated the interaction of a cationic detergent, dodecylammonium chloride, with ovalbumin. In all cases the mobility of the protein was shifted considerably in the presence of the detergent, indicating binding. In the presence of an excess of protein, boundaries corresponding to free protein and complex could be observed (97, 100); the free-protein boundary was found to disappear as the amount of detergent was increased, with one or more boundaries (97) due to complexes appearing. In most cases, interaction of proteins with detergents resulted in some permanent changes in the protein molecule, especially in the presence of high detergent concentration. This aspect of the problem will be discussed later.

B. *Protein-Protein Interactions*

The theory of protein-protein interactions is discussed in detail in Chapter 3, Sections VI and VII. A classification of various types of interactions is given there, and criteria for distinguishing between them are presented.

One of the most elegant physical studies of protein-protein interaction is the work of Singer and co-workers (101–107) on the soluble complexes of protein antigens with the corresponding antibodies.

Antibodies and antigens are substances capable of interacting specifically with one another in proportions which depend on the composition of the mixture. It has been customary to call the number of combining sites on each protein the valence of that protein. The work of Pauling and co-workers (108, 109) suggests that the precipitating antibodies are divalent and antigens are multivalent. Goldberg (110) has shown that such a system would have a zone of precipitation but that soluble complexes would exist under certain conditions, such as the presence of a large excess of antigen.

Singer and co-workers used the ultracentrifuge and electrophoresis to study the soluble antigen-antibody complexes found in the presence of excess antigen.

The antigens studied were bovine serum albumin (BSA) (101), ovalbumin (102, 103), hapten-substituted serum albumin (104, 107), chemically modified serum albumin resulting in a univalent antigen (105), β-lactoglobulin, and conalbumin.

Electrophoretic diagrams obtained on the BSA-anti-BSA system with different ratios of antigen to antibody in a pH 8.5 barbital buffer of 0.1 ionic strength are shown in Fig. 10. It is possible to see that there is no free γ-globulin present. The most rapid peak is the free antigen (BSA), while the other peaks represent complexes.

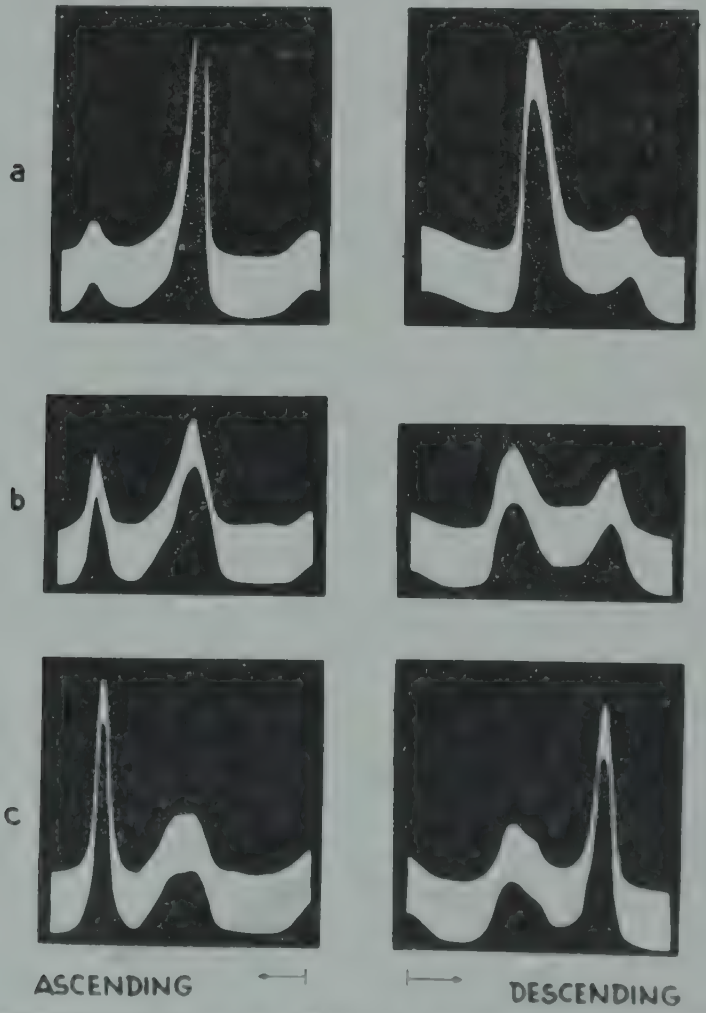


Fig. 10. Electrophoresis diagrams of soluble antigen-antibody complexes (101) at the following values of (AG/AB), total protein concentration and times of migration respectively: a, 0.56, 18.0 mg./ml., 7740 seconds. b, 1.00, 16.1, 7800 seconds; c, 2.14, 16.5, 7560 seconds.

The complexes which exist in antibody-antigen systems constitute an equilibrium system, and the results one obtains in the electrophoresis apparatus are strongly dependent upon the rate at which equilibrium is established. Singer and Campbell (101) have set up some criteria for the quantitative use of schlieren diagrams for systems which may undergo re-equilibration.

In the ascending boundary we have Ag (free antigen) and two complexes Ag_2Ab (*a* complex) and Ag_3Ab_2 (*b* complex). The three components have mobilities $-u_A > -u_a > -u_b$. Figure 11 shows the boundary system in the ascending limb of the electrophoresis apparatus. In phase α behind the A boundary, there is only free antigen and no reaction takes place. In phase β, we have then the *a* complex and the disproportionation of Ag_2Ab may take place:

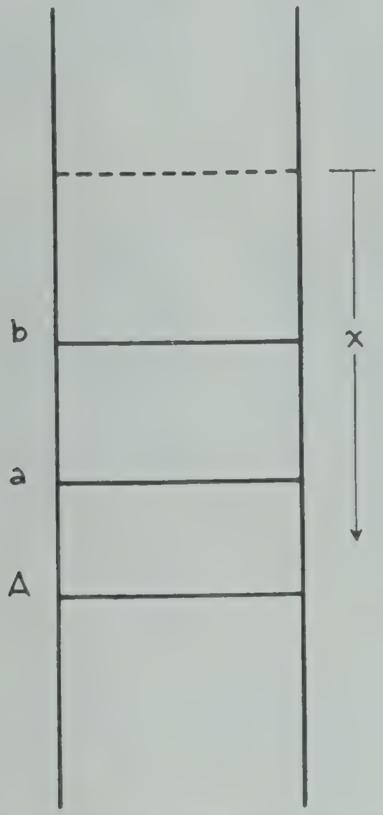


Fig. 11. Moving boundary system in the ascending limb of the Tiselius cell for the bovine serum albumin-Antibovine serum albumin system (101)

$$2Ag_2Ab \xrightarrow{R} Ag_3Ab_2 + Ag \tag{18}$$

R is the over-all rate constant of this reaction. At some later time, the

ΔAg produced in B will overtake the a boundary and be found between A and a. The additional amount, ΔAg, of the antigen found in phase α as the result of this reaction will be

$$\Delta \mathrm{Ag}(x_1 t) = \frac{R_x x(u_a - u_b)}{(u_a - u_{\mathrm{A}})(u_b - u_{\mathrm{A}})} > 0 \tag{19}$$

It follows that $d(\Delta \mathrm{Ag})/dx$ is positive and that the schlieren curve in the α phase will be elevated above the baseline. If, on the other hand, the schlieren curve in this region does return to the baseline, then the effects of re-equilibration are negligible and the area under the A peak on the ascending side will correspond to the concentration of free antigen present in the original solution. On the descending side, the distribution of components and the resulting shifts in equilibrium are quite different. As a result, the patterns do not return to the baseline between the peaks for the a complex and the free antigen.

Careful analysis of the electrophoretic data (101) has made it possible to obtain the equilibrium concentration of uncombined antigen in a solution of known total antigen and total antibody content. For this purpose boundary anomalies were properly taken into account by running a number of control experiments on noninteracting systems. Plotting the amount of antigen combined per antibody molecule against the relative concentration of antigen, the ratio of antigen to antibody in complexes extrapolates to a value of 2 at 100% antigen, showing the antibody valence to be 2.

An extension of these studies as a function of pH resulted in a pH-mobility curve for BSA in the complexed system identical with that of pure BSA, showing again that the effects of re-equilibration are not important. From a comparison of calculated to observed mobilities of the complexes at various pH's, further evidence was obtained for the bivalence of antibodies. Applying the Goldberg theory, equilibrium constants were calculated for the $\mathrm{Ag} + \mathrm{AgAb} \rightleftharpoons \mathrm{Ag_2Ab}$ reaction. The values obtained were $K = (2.5 \pm 0.5) \times 10^4$; $\Delta F° = -5.5 \pm 0.2$ kcal.; $\Delta H° = 0 \pm 2$ kcal.; $\Delta S° = 20 \pm 8$ entropy units, in barbital buffer, pH 8.5 at 0°. Similar values were obtained with other proteins.

A plot of the pK for the $\mathrm{Ag} + \mathrm{Ab} \rightleftharpoons \mathrm{AgAb}$ reaction versus pH resulted in a straight line with unit slope, showing that a single ionized group is involved in every Ag Ab bond, both in the anti-BSA and anti-ovalbumin systems.

By acetylation and guanidination of the BSA and the anti-BSA antibody, Singer (106) has shown further that there is one amino group per reactive site of the anti-BSA antibody molecule. In order to show that loss of activity is due to chemical reaction at the antibody reactive sites and not to nonspecific effects, electrophoretic experiments were carried out on a solution of acetylated BSA-anti-BSA complex and a mixture of separately acetylated BSA and rabbit γ-globulin. The electrophoretic patterns, shown in Fig. 12, clearly demonstrate that activity is retained in the first case only.

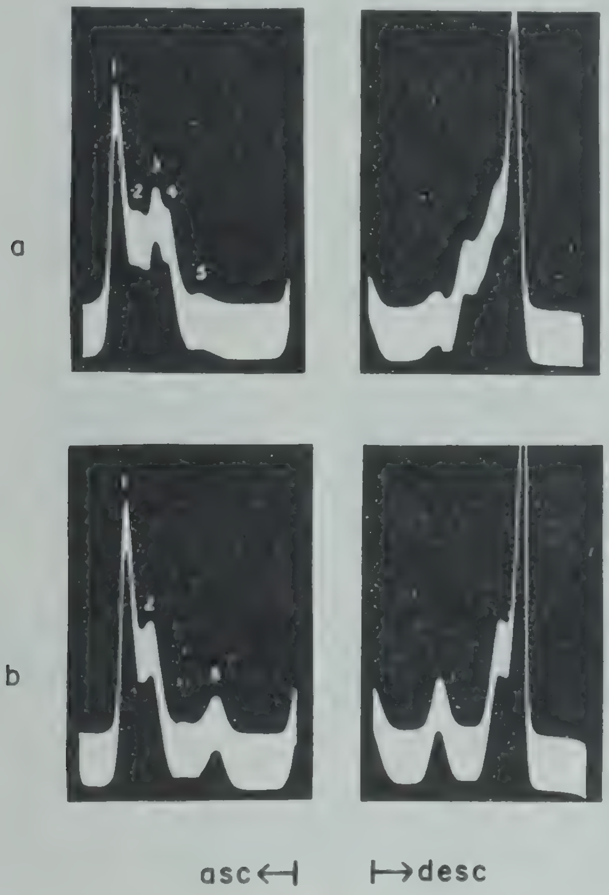


FIG. 12. Electrophoretic patterns of (a) acetylated BSA-anti-BSA complexes and (b) a mixture of separately acetylated BSA and rabbit γ-globulin in veronal buffer (pH, 8.68; $\Gamma/2$, 0.1). Boundaries 1 and 2 are due to acetylated BSA, boundaries 3 and 4 to AC-AE complexes, and boundary 5 to acetylated γ-globulin (106).

Another type of interaction between biologically important proteins is that of enzymes with specific inhibitors. Ram and associates (111) have studied electrophoretically the stoichiometry of the reaction between trypsin and its inhibitor, ovomucoid. Using an analysis of the data similar to that of Singer and co-workers, these authors found that while trypsin can bind only one ovomucoid molecule, more than one trypsin can react with each molecule of ovomucoid. The isoelectric points of the two proteins and their equimolar complex are 10.8, 4.3, and 9.0, respectively. This can be explained on the basis of the number of ionizable groups on the two proteins in this pH range. In this case also, the presence of the complexes does not affect the mobilities of other components present in the system, and the ascending pattern returns to the baseline between peaks, showing that re-equilibration effects are not significant.

Inactivation of trypsin with the DFP reagent results in loss of its ability to complex with ovomucoid as shown by Chernikov and Shpikiter (112). Their electrophoretic data, shown in Fig. 13, are considered to be strong evidence in favor of the theory that the inhibitor is actually bound by the active site of the enzyme.

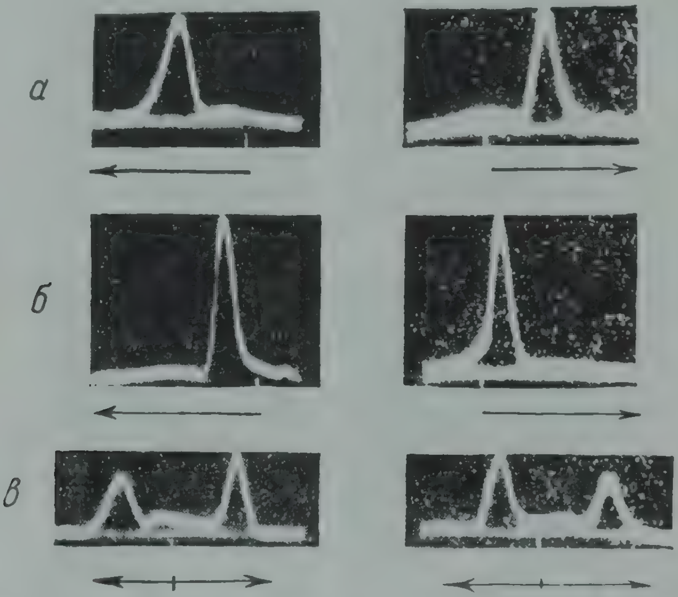


FIG. 13. Electrophoretic diagrams of the trypsin-ovomucoid system (112). a, ovomucoid; b, 1:1 mixture of trypsin and ovomucoid; c, 1:1 mixture of DP-trypsin with ovomucoid. Right: descending; left: rising. 180 Minutes in pH 7.15 veronal buffer of 0.2 ionic strength, at 3.5 volts per centimeter.

Similar data on the complexing of chymotrypsin by ovomucoid have been obtained by Weil and Timasheff (unpublished work, (112a)).

An interesting study of the complexing of two proteins has been carried out by Ehrenpreis and Warner (113) on the conalbumin-lysozyme system. In this case, the difference in the sizes of the molecules has made it possible to compare the electrophoretic data with dialysis equilibrium results. This system was found to be of the rapid re-equilibrating type (see Chapter 3, VI, A). The fact that the two proteins were oppositely charged under the experimental conditions made analysis of the data possible. The reaction was interpreted in terms of the consecutive association of two lysozyme (L) molecules with one of conalbumin (C):

$$\begin{aligned} L + C &\rightleftharpoons CL \\ CL + L &\rightleftharpoons CL_2 \end{aligned} \tag{20}$$

The constituent mobilities, obtained experimentally as described in Chapter 3, were defined as:

$$\begin{aligned}(\mathrm{C})\,\bar{u}_{\mathrm{c}} &= (\mathrm{C})\,u_{\mathrm{c}} + (\mathrm{CL})\,u_{\mathrm{cl}} + (\mathrm{CL}_2)\,u_{\mathrm{cl}_2}\\ (\mathrm{L})\,\bar{u}_{\mathrm{l}} &= (\mathrm{L})\,u_{\mathrm{l}} + (\mathrm{CL})\,u_{\mathrm{cl}} + 2(\mathrm{CL}_2)\,u_{\mathrm{cl}_2}\end{aligned} \tag{21}$$

The corresponding equations for the constituent concentrations are:

$$\begin{aligned}(\overline{\mathrm{C}}) &= (\mathrm{C}) + (\mathrm{CL}) + (\mathrm{CL}_2)\\ (\overline{\mathrm{L}}) &= (\mathrm{L}) + (\mathrm{CL}) + 2(\mathrm{CL}_2)\end{aligned} \tag{22}$$

where the u_i's are the mobilities of the corresponding species. Since at the experimental pH, conalbumin and lysozyme have about equal but opposite charges, u_{cl} is small, and since (CL_2) is also small, $(\mathrm{CL})\,u_{\mathrm{cl}} + (\mathrm{CL}_2)\,u_{\mathrm{cl}_2}$ in equation (21) could be neglected. This made possible the analysis of the electrophoretic data, which turned out in good agreement with dialysis equilibrium results. In cases in which the last two terms in equation (21) are sufficiently large so that they cannot be left out, quantitative analysis becomes impossible, since there are more unknowns than equations.

The system ovalbumin-yeast nucleic acid (114) has also received a detailed quantitative study. This is discussed in Chapter 3, Section VI, B. Similar studies have been carried out on the interaction of ovomucoid with yeast nucleic acid (114), of serum albumin with thymus nucleic acid (115) and with fish sperm nucleic acid (116), as well as of insulin with protamine (117). Longsworth and co-workers (37) have pointed out the interaction of various components of egg white. Mixtures of α- and β-casein (118), as well as of the various components of gliadin (119), also display electrophoretic evidence of interaction.

C. Polymerization Reaction

A number of systems exist in which the protein aggregates reversibly under a given set of conditions. For the case in which the re-equilibration is rapid, Gilbert (120) has shown that for the reaction $nB \rightleftharpoons B_n$, $K = (B)^n/(B_n)$, if n is greater than 2, the refractive index gradient curve may have two maxima. For a dimerization reaction only one boundary will be present. Using this theory, which is discussed in detail in Chapter 3, Section VII, B, it is possible to determine the equilibrium constant of the reaction, if the extent of aggregation (n) is known.

A number of protein systems are known which undergo a polymerization reaction. Among those that have been studied electrophoretically are arachin (121), chymotrypsin (122), and chymotrypsinogen (122). The most thoroughly studied polymerizing system is β-lactoglobulin (23, 72, 75, 123-125). Ogston and Tilley (75), in comparing electrophoretic experiments with some ultracentrifuge measurements showed that the electrophoretic heterogeneity of that protein at pH 4.65 is due to polymerization. Townend and Timasheff (79) showed this association to be limited to the pH region between 3.5 and 5.25 with maximal association taking place between pH 4.40 and 4.65. Following the discovery by Aschaffenburg and Drewry (76) that this protein consists of two genetically different entities, Ogston and Tombs (123) found that the electrophoretic patterns of the two are different in the pH region of association, with the conclusion that β-lactoglobulin *A* probably associates to a much greater extent than β-lactoglobulin *B*. A comparison of electrophoretic and ultracentrifugal patterns for these two proteins was carried out by Townend and Timasheff (125). Typical patterns are shown in Fig. 14. It can be seen that, whereas both proteins give complex electrophoretic patterns, only *A* shows evidence of association in the ultracentrifuge.* Light-scattering studies by Townend and Timasheff (125) have shown that the re-equilibration is very rapid (less than 1 minute) and that the polymer formed is most probably a tetramer. Using this information, as well as Tombs' report (124) that only 90% of the A protein can give the complex electrophoretic patterns, Townend and Timasheff calculated an equilibrium constant for the re-

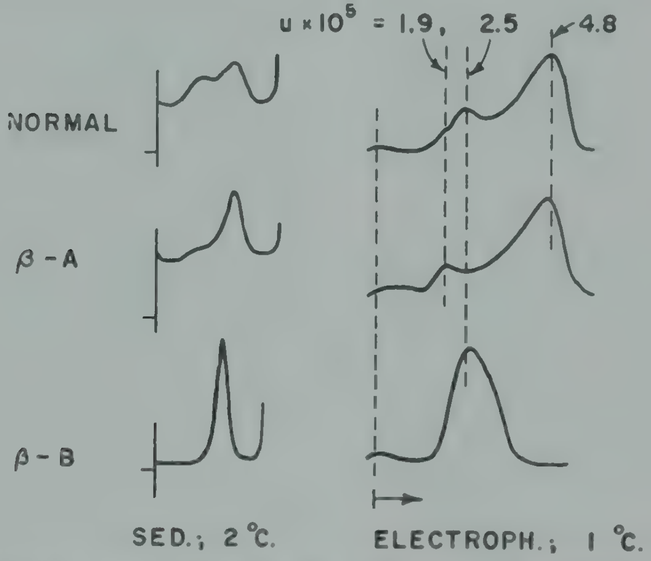


FIG. 14. Tracings of ultracentrifugal and electrophoretic patterns (descending) of various β-lactoglobulins in pH 4.65 acetate buffer, $\Gamma/2 = 0.1$ (125). Both sedimentation and electrophoretic migration proceed from left to right. Sedimentation: 59, 780 r.p.m.; "normal" and β-A, 1.4% protein, 160 minutes; β-B, 7% protein, 352 minutes. Electrophoresis: 1.6% protein, 8000 seconds at 9.7 volts per centimeter.

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* Klostergaard and Pasternak (78) have come to the opposite conclusion.

action $4\beta^{A'} \rightleftharpoons \beta_4^{A'}$ with the aid of the Gilbert theory (120). The equilibrium calculations were done in the following manner. For $n = 4$, the function δ at the minimum point of the pattern is $\delta = (n - 2)/(3n - 3) = 0.222$. From the experimental data on the area distribution at a series of protein concentrations, one can calculate the concentration of polymerizable protein at which 50% will be aggregated, since above that concentration the fraction of the slow peak due to the polymerizable protein must have a constant area at all concentrations (120). In the case of β-lactoglobulin *A*, when correction of the areas was made for 10% nonpolymerizable material, it was found that the slow peak at the concentrations studied corresponded to 0.80 ± 0.20 gm. per liter of protein monomer. A comparison of calculated and observed area distributions under the two peaks of the reaction boundary on the descending side is given in Table II.

TABLE II

ASSOCIATION OF β-LACTOGLOBULIN A[a,b]

Protein conc. g/1	% Major component Observed	% Major component Calculated[c]
5.2	71	74
10.0	79	82
16.0	86	85
16.3	85	85
20.4	87	86
12.8[d]	91	93

[a] According to Townend and Timasheff (28).

[b] 90% aggregable material (124). Area distribution in electrophoresis in pH 4.65 acetate of 0.1 ionic strength, 1°C.

[c] Calculated according to Gilbert theory (120); $K = 7.8$ gm.^{3} -1^{-3}; $n = 4$.

[d] Purified aggregable fraction.

Using the value of 0.80 gm. per liter for the constituent concentration, $\bar{m}$, at the minimum point and the corresponding δ value for $n = 4$, K was found to be equal to 7.8 ± 3.4 gm.^{3} $-$ 1^{-3}. This results in a standard free energy of association of $\Delta F^\circ = -15.4 \pm 0.5$ kcal. per mole at 1° for the tetramerization of the polymerizable fraction of β-lactoglobulin *A*. These values of K and ΔF° are in good agreement with corresponding values obtained from light scattering and ultracentrifugal data. It might be of interest to note that application of the Gilbert theory to ultracentrifugal data on the polymerization of β-lactoglobulin at pH 4.65 (125), as well as to its dissociation into half-molecules at pH 1.6 (126, 127), made it possible to calculate K values in good agreement with light-scattering data, as well as the concentration dependence of the sedimentation constant.

D. Isomerization Reaction

In Chapter 3, Longsworth described qualitative criteria for recognizing boundaries in which re-equilibration of isomers occurs. Cann and associates (128) have made a theoretical study of the electrophoretic behavior of systems containing a protein which can exist in two interconvertible states, A and B.

In treating the problem, it was assumed that the states A and B have mobilities μ_1 and μ_2 and diffusion constants D_1 and D_2. The specific rates of interconversion are k_1 and k_2,

$$\mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{B} \qquad (23)$$

and the concentrations of A and B are represented by C_1 and C_2. During electrophoresis of this system, the changes in concentrations with time of electrophoresis, t, and height in the electrophoresis cell are described by the following set of forced-diffusion equations where the position variable, x, is measured relative to a frame of reference moving at velocity $\mu_1 Et$ and E is the field strength.

$$\text{(a)} \qquad \frac{\delta C_1}{\delta t} = D_1 \frac{\delta^2 C_1}{\delta x^2} + k_2 C_2 - k_1 C_1$$

$$\text{(b)} \qquad \frac{\delta C_2}{\delta t} = D_2 \frac{\delta}{\delta x}\left[\frac{\delta C_2}{\delta x} - \frac{\gamma E C_2}{kT}\right] - k_2 C_2 + k_1 C_1 \qquad (24$$

$$\text{(c)} \qquad \gamma = \frac{z_2 e \mu_2 + z_1 e \mu_1}{\mu_2};\ \mu = ze\mu;\ D = nkT$$

The symbol z represents the net electrical charge and e the electronic charge.

If the concentrations are represented by their Fourier integrals,

$$C(x_1 t) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} G(\sigma_1 t) e^{-i\sigma x}\, d\sigma \qquad (25)$$

the equations (24) can be solved for G_1 and G_2. C_1 and C_2 may then be obtained by evaluation of the Fourier integral (25). Analytical solution of the Fourier integrals for the general case is extremely difficult. However, an approximate analytical solution can be obtained for the special case where $k_1 = k_2 = k$ and $D_1 = D_2 = D$. If it is assumed that k is sufficiently large, so that $4k^2 \gg (\sigma\gamma' E)^2$ where $\gamma' = \mu_2 - \mu$, then the electrophoretic gradient curve is given by the relation

$$\frac{-\delta(C_1 + C_2)}{\delta x} = \frac{C_{20}}{\sqrt{\pi}} e^{-(\frac{1}{2}\gamma' Et - x)^2/4Dt} \left\{ \frac{1}{(Dt)^{1/2}} - \frac{\gamma'^2 E^2 e^{-2kt}}{32k^2 (Dt)^{3/2}} + \frac{\gamma'^2 E^2 e^{-2kt}}{64k^2 (Dt)^{5/2}} (\tfrac{1}{2}\gamma' Et - x)^2 \right\} \quad (26)$$

where C_{20} is the concentration of component B at zero time. It can be seen by inspection that in the limit of infinitely large rates of reaction, where equilibrium would be expected to be maintained during electrophoresis despite differential migration of the components, equation (26) predicts that the system as a whole will migrate as a homogeneous substance. On the other hand, bimodal boundaries are predicted for rather slower rates of reaction.

Some illustrative calculations have been made using equation (26) expressed in terms of the reduced variables $x' = x/l$, $t_0 = kt$ and $\alpha = D/kl^2$ where l is a characteristic length equal to $\gamma' E/k$. Theoretical gradient curves at several values of the reduced time, t_0, for each of two values of k are shown in Fig. 15. These calculations indicate that resolution into two boundaries will occur for times of electrophoresis less than, or of the order of, the half-time of reaction, but that for longer times a single moving boundary spreading by diffusion only will be observed.

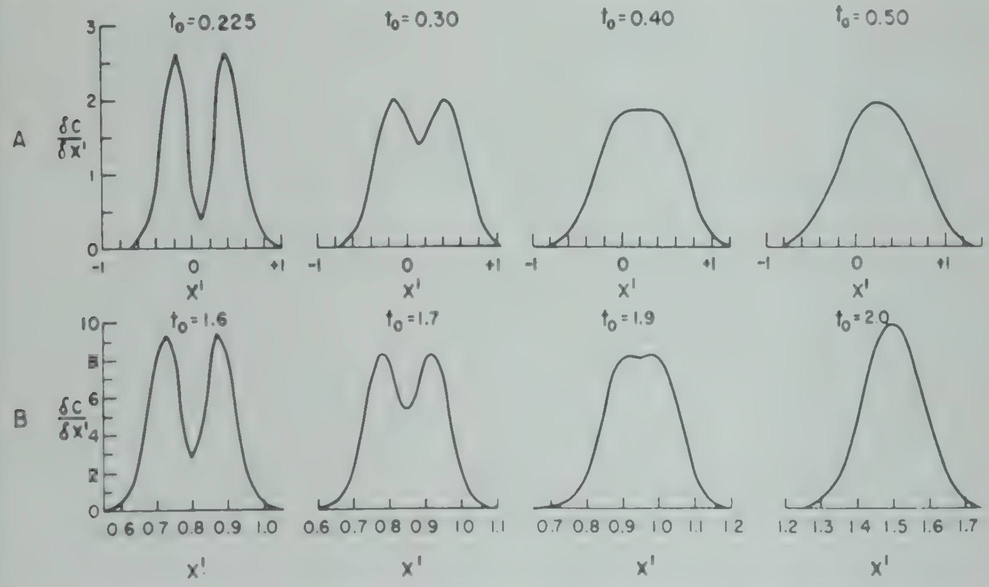

FIG. 15. Illustrative calculation of electrophoresis gradient curves using equation (26): A, $\alpha = 10^{-1}$; B, $\alpha = 10^{-3}$ (128).

The theory of isomerization equilibrium in electrophoresis presented above is obviously correct only within the limits of its assumptions. It should provide a valuable guide in the interpretation of electrophoretic data. However, its limitation should be recognized. It does not discuss the possibility and consequences of pH, salt, and density gradients which may exist across the boundaries. The more complicated reactions involving more than one molecule are beyond the scope of the theory.

E. The Behavior of Proteins in Acid pH's

The subject of equilibrium systems in electrophoresis has been receiving considerable attention lately because of the interest in the behavior of proteins below pH 4.5. While a number of protein systems have been examined, bovine serum albumin has become more or less the standard, and most of the studies have been done with this protein.

Bovine serum albumin normally travels as one peak in the electrophoresis apparatus above pH 4.5. Below this pH more than one peak is observed and the patterns are usually nonenantiographic. Furthermore, the pH range of electrophoretic heterogeneity is found to be a function of the buffer used.

The first observation on the electrophoretic heterogeneity of serum albumin was made by Luetscher (129), who found that crystalline human and horse serum albumins show two electrophoretic boundaries in acetate buffer of pH 4.0 and ionic strength 0.02. Sharp and co-workers (130) reported that horse serum albumin displayed a complex boundary pattern in the pH range between 4 and 6 both at 0.1 and 0.02 ionic strength. These patterns were distinguished by a lack of symmetry between the ascending and descending boundaries, which was particularly pronounced at pH near 4.4. Longsworth and Jacobsen (23) concluded that the nonenantiographic patterns of this protein indicate continuously readjusted equilibria across the moving boundaries. The heterogeneity of serum albumin in acid pH's has been observed also by a number of other workers (131–133).

The problem of the heterogeneity of BSA in acid pH's recently has undergone broad investigations by Aoki and Foster (134–139) and by Cann and Phelps (140–143).

Aoki and Foster have examined the electrophoretic behavior of BSA in the pH range of 2 to 5 using 0.2% protein solutions at 0.02 ionic strength of chloride, thiocyanate, and acetate. In the pH region between 3.0 and 4.5, two boundaries were observed, the percentage composition changing continuously with pH. At 0.2% protein, the patterns were found to be fairly enantiographic, while at higher concentration, enantiography was lost. These authors explained the anomalous behavior of bovine serum albumin in the electrophoresis apparatus at pH 4.0 by an isomerization reaction of serum albumin involving a change in the intrinsic ionization constants of the carboxyl groups of the protein. The observations were explained in terms of the equilibrium

$$N + 3\mathrm{H}^+ \rightleftharpoons F$$

The titration data for BSA are also anomalous below pH 4.5 (144). Aoki and Foster accounted for the titration data obtained between pH 3.5 and 4.5 by assuming that the carboxyl groups of the two forms have different intrinsic ionization constants, with the N form having a $\mathrm{p}K_0$ value of 3.7 and the F form, 4.4. Assuming that the area under the fast-moving peak in the electrophoresis apparatus represents the concentration of the F form and that the area under the slow-moving peak represents the N form, Aoki and Foster calculated the pH-dependence of the distribution of the two forms. This is shown in Fig. 16, and is in good agreement with their interpretation of the titration data.

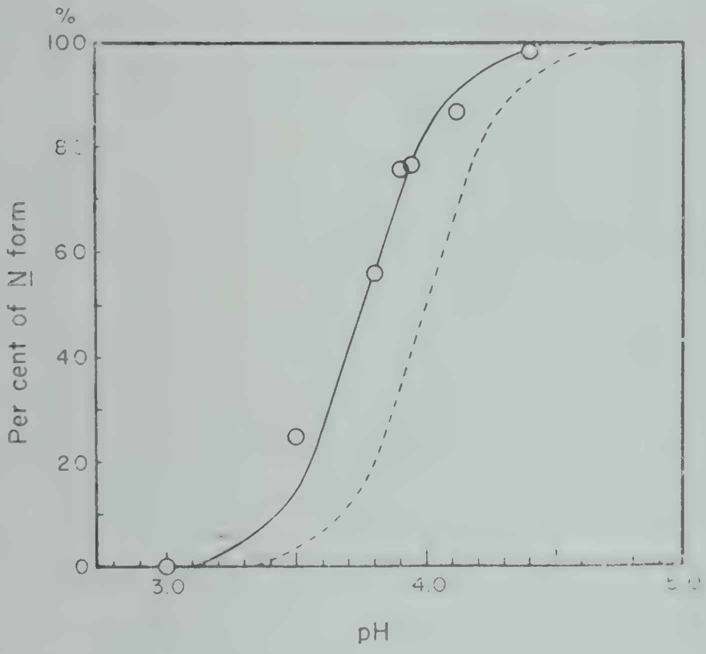

FIG. 16. Dependence of electrophoretic composition of bovine serum albumin on pH at 0°C., 0.2% protein. — 0.02 ionic strength SCN^-; – – – 0.02 ionic strength Cl^- (136).

Further investigations of this problem under the same conditions, as well as in the presence of urea (138) and detergent ions (139), have given evidence that the composition and the mobilities of the components are constant over a wide variety of lengths of runs, indicating re-equilibration to be not important. In the interpretation of the data, however, Foster and Aoki had to assume that the equilibrium represents the sum of four cooperative transitions (137) in order to reconcile quantitatively the electrophoretic and titration information.

It has not been established unequivocally that the electrophoretic patterns obtained with BSA in the pH region of 3.5 to 4.5 can be interpreted in this simple manner. Certain difficulties are yet to be overcome. Correlation of the titration data with electrophoresis requires knowledge of ion binding. Unfortunately, the information on the binding of chloride and thiocyanate ions as a function of pH is as yet incomplete. Furthermore, the assumptions of four cooperative transitions are yet to receive independent experimental validation. One should also point out that a serious discrepancy exists between the experimental reaction velocity and the predictions of the theory of Cann *et al.* (128). Thus, until further experimental and theoretical developments become available, the above interpretation of the data will remain open to question and should be considered only as a very interesting model for such protein behavior. It should be pointed out that in a recent publication, Bro and Sturtevant (145) have reported on calorimetric evidence showing that a transition is occurring in BSA in the pH region of 3.5 to 4.5, with a heat of reaction similar to that calculated by Aoki and Foster (135) from electrophoretic data and a half-time of about 2 minutes. Furthermore, Tanford and associates have concluded from titration and viscosity data that below pH 4.3, BSA undergoes a change from a compact to an expandable form (144).

Cann and Phelps (140–143) have carried out similar studies of the electrophoretic behavior of BSA, ovalbumin, and γ-globulin. Working at pH 4.0 in low and high ionic strength buffers, at protein concentrations of approximately 1.3%, these authors have observed that the three proteins display complex nonenantiographic patterns. (The work with γ-globulin was carried out at pH 5.7 and 4.0.) Electrophoretic patterns obtained in acetate-chloride buffers of various compositions are shown in Fig. 17. As can be seen, progressive changes in the electrophoretic patterns occur as the concentration of NaAc-HAc increases. These were found to be very sensitive to the NaAc-HAc concentration, but not to ionic strength, if the acetate concentration remained constant. Comparison of data obtained in a series of carboxylic acid buffers showed a close correlation with the concentration of nonionized carboxylic acid in the supporting medium and a very poor one with the acid anion. A similar study in the presence of formic acid showed the electrophoretic patterns to be strongly pH-dependent, suggesting the participation of carboxylate groups on the protein in this reaction. This is consistent with the finding of these authors that the electrophoretic patterns of methylated BSA at pH 4.0 are insensitive to the concentration of the acetate buffer (145a).

A tentative qualitative interpretation of this behavior can be given in terms of the binding of undissociated acid molecules by the protein with

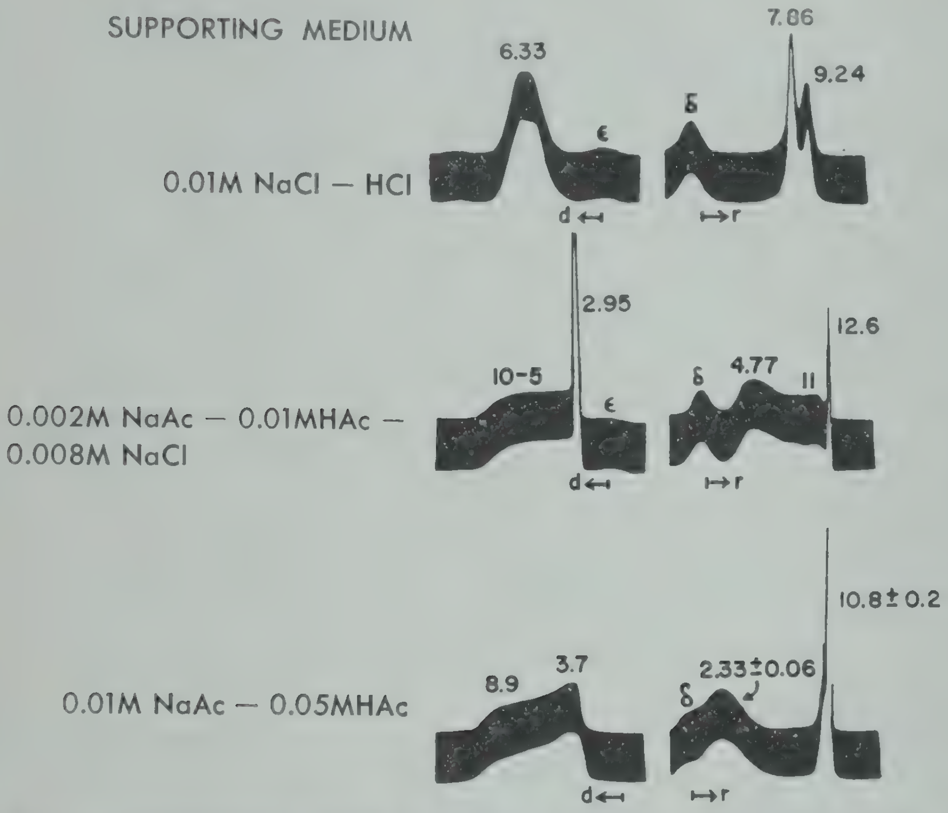

Fig. 17. Electrophoretic patterns of 1% BSA in various supporting media at pH 4.0. Boundary velocities ($10^5 \times u$) are shown above or beside corresponding peaks (143).

the assumption that the resulting complex has a higher mobility than the original protein (145a):

$$P \text{ (slow)} + \text{HAc} \rightleftharpoons P\text{HAc (fast)}$$

The electrophoretic pattern can then be explained with the help of the boundary diagram shown in Fig. 18. On the descending side, the *P*HAc form moves with its normal mobility into the original equilibrium mixture, leaving behind *P*. In order to readjust equilibrium, *P* reacts with HAc from the buffer, lowering the HAc concentration behind the *P*HAc boundary and raising the pH in that region. This causes a retardation in the migration at the front of the slow *P* boundary and results in sharpening, since the rear portion of the boundary migrates in the original lower pH buffer. The descending limb should then consist of a leading broad *P*HAc boundary, followed by a sharp slow boundary, with the pH between the two higher than the original pH. Separation into two boundaries may occur even if the reaction is very rapid as shown by Gilbert and Jenkins (146). On the rising side, the rapidly migrating *P*HAc moves out into fresh buffer. Due to re-

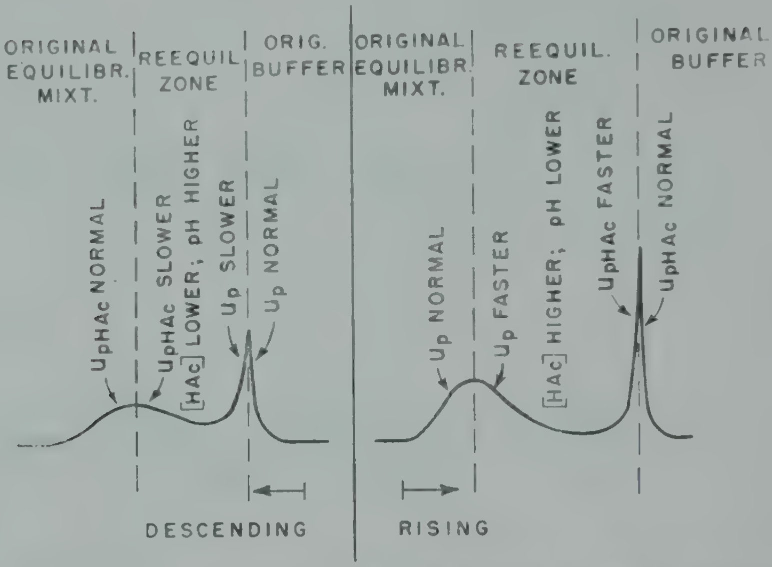
ORIGINAL EQUILIBR. MIXT.
REEQUIL ZONE
ORIG. BUFFER
UpHAc NORMAL
UpHAc SLOWER
[HAc] LOWER; pH HIGHER
Up SLOWER
Up NORMAL
DESCENDING
ORIGINAL EQUILIBR. MIXT.
REEQUIL. ZONE
ORIGINAL BUFFER
Up NORMAL
Up FASTER
[HAc] HIGHER; pH LOWER
UpHAc FASTER
UpHAc NORMAL
RISING

Fig. 18. Model boundary system that could account for effects of acetic acid binding on electrophoresis of BSA in pH 4.0 acetate buffer.

equilibration, HAc is liberated behind the boundary, lowering the pH behind the boundary and causing its sharpening. The boundary, due to slower-migrating *P*, moves into the zone of lower pH, its leading edge becoming accelerated, while its tailing edge migrates in the original buffer. This would result in a pattern consisting of a sharp, rapid boundary, followed by a slow, broad one, with the pH between the two being lower than in the original solution.

Camm has measured the actual pH and conductivity at various points on the rising side and compared these values with those calculated from the Dole theory. It is of interest that while the changes in conductivity, observed behind the various boundaries, can be accounted for in terms of the Dole theory, the pH changes were always found to be significantly greater than those calculated and in qualitative agreement with those expected from the above-described mechanism. Thus, it would appear that at higher protein concentrations, although protein disappears across the various moving boundaries, these may be considered as false boundaries in the sense that they correspond neither to single stable protein components nor to single components involved in a slowly adjusted equilibrium. These false boundaries do not arise solely as a result of the establishment of conductance and pH gradients in the electrophoresis cell, but are associated with complexing of the protein with buffer constituents, in particular with the undissociated buffer acid.

Phelps and Camm (142) have found further that introduction of amino acids into the buffer system results in changes that could be interpreted as competitive binding of the amino acid to the same sites as the carboxylic acid. The active form in this case is the acid form of the amino acid rather than the zwitterion, and steric factors are important in its action.

Since the theory of the effect of binding of neutral molecules on the electrophoretic behavior of a protein has not been developed yet, a complete understanding of these observations is not possible.

Observations, similar to those described above, have been made by Singer and Campbell (101), who have found that in glycine-HCl buffers of pH 2.35 and 0.1 ionic strength, BSA, γ-globulin, β-lactoglobulin, ovalbumin, and conalbumin all give two peaks in both sides of the electrophoresis cell. Experiments were carried out on BSA by Dintzis, Timasheff, and Singer (146a) in which the rapid peak was isolated from the rising side of the Tiselius cell and the slow peak from the ascending side. Subsequent electrophoresis at the same conditions of the two isolated "components" showed a picture identical with the original pattern no matter how rapidly the sample was removed and rerun. This observation is identical with those of Cann and Phelps (141, 142) on BSA and ovalbumin at pH 4.0, who also obtained patterns identical with the original material on rerunning samples withdrawn from the Tiselius cell.

Recently, the problem of protein heterogeneity in the presence of amino acids has been extended by Woods (147), who examined BSA, ovalbumin, lysozyme, bovine γ-globulin, bovine fibrinogen, a water-soluble gelatin, and poly-L-lysine in various buffers between pH 1.5 and 3.0. In all cases, Woods found that when the buffer contained an amino acid hydrochloride, patterns similar to those observed by Singer and Campbell (101) were obtained, while normal patterns were found in other buffers. Analysis of this phenomenon led to the conclusion that the behavior of these boundaries does not fit the requirements of a false boundary of the Svensson type (26). Woods (147) suggests that the patterns can be interpreted qualitatively in terms of the moving boundary theory of weak electrolytes. It is of interest, however, that, if it is assumed that the glycinium form of the amino acid is bound to the protein with the formation of a more rapidly migrating species, it is again possible to account qualitatively for this behavior in terms of the boundary pattern used with BSA in the presence of NaAc-HAc (Fig. 18). Indeed, the pH changes expected across the various boundaries in such a system are in qualitative agreement with those determined by Woods (147) in both the rising and descending limbs of the cell.

Nonenantiographic patterns similar to those of Cann and Phelps, have been observed on BSA by Schilling (91) in 0.1 ionic strength NaAc and cadmium acetate buffers at pH 6.0.

A very interesting finding is that of Schmid (148), who prepared a serum albumin which gave normal patterns in acetate buffer at pH 4. After treatment with cysteine and an ion exchanger, however, nonenantiographic patterns, similar to those described above, were obtained. Schmid suggests that cysteine removed a blocking agent from the protein (148), permitting it to enter into the isomerization and acid-binding reactions described above.

The question of the exact nature of the equilibrium mixtures observed in the electrophoresis apparatus at low pH is still open. Recent experiments by Cann (143) on the effect of protein concentration on the electrophoretic patterns of BSA in 0.01 Ac^- 0.01 Cl^- at pH 4.0 indicate that the effects, observed by Aoki and Foster (134–139) and by Cann and Phelps (140–143), are different. It was found that the slow boundary, observed by Cann and Phelps, arises gradually as the protein concentration is increased from 0.2 to 1%. As shown in Fig. 19, at 0.2% only the two components studied by Aoki and Foster are present, while at 1%, the pattern becomes much more complex. Thus, in the present state of knowledge, one might make the

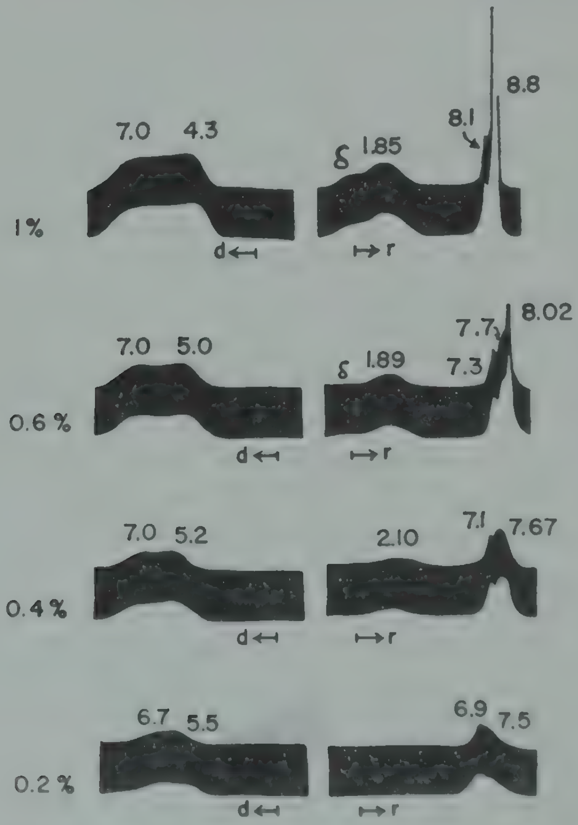

FIG. 19. Electrophoretic patterns shown by BSA at various protein concentrations in 0.01 *M* NaAc, 0.05 *M* HAc, – 0.01 *M* NaCl, pH 4.0. Boundary velocities ($10^5 \times$ are shown above or beside corresponding peaks (143).

tentative conclusion that in the pH region close to 4, two distinct phenomena occur in BSA; one is a transformation of the protein, best observable at low concentration, the other is binding of buffer acid, which leads to complex electrophoretic patterns.

The interpretation of the electrophoretic data might be somewhat easier if more information were available on the pH and conductivity changes across the boundaries in the various systems studied. It seems probable, however, that the observed electrophoretic behavior of proteins in acid pH's is the result of the cooperative influence of true heterogeneity, isomerization, and the binding of ions and small molecules. Thus, a great deal of work remains to be done before this question is fully elucidated. The mechanisms presented above can serve as possible hypotheses that might account for the observations. In no case should they be considered as final interpretations of the data.

F. Protein Interactions

While the theories of various types of reactions that can occur in proteins are far from completely developed, a great deal of progress has been made in the understanding of patterns found in reacting systems. Only a few reaction cases are known where the pattern is enantiographic. These are the protein-detergent systems (96, 97, 100), the antigen-antibody system (101–105), the trypsin-ovomucoid interaction (111, 112) and the BSA study in acid pH at low concentrations (134, 135, 138, 139). In some of these cases the boundaries may be interpreted as representing single species of protein or complex, and their areas and mobilities can be considered as a first approximation to correspond to those of discrete components. In most cases, however, the patterns are strongly nonenantiographic and great care has to be exerted in their proper interpretation. A great deal more of theoretical work is necessary, however, especially on the effects of salt, pH, and density gradients on the reaction boundaries, and on the binding of neutral molecules to proteins, as well as on the moving boundary theory of weak electrolytes, before a complete understanding of electrophoretic systems in equilibrium can be reached.

As a further word of caution on the interpretation of electrophoretic patterns obtained in interacting systems, it must be pointed out that many of the proteins, which are considered to be "pure," have been found recently to be resolvable into several components by various techniques, such as chromatography. As an example, it is sufficient to cite the work of Tiselius and associates, who separated BSA into three distinct components (149). Since various components of a "pure" protein may exhibit different behavior in interactions and isomerizations, the actual systems may be much more complicated than is realized at present. One can cite as examples the electrophoretic difference at pH 4.0 between BSA as prepared by Schmid and standard BSA preparations, as well as the difference in association properties of β-lactoglobulins *A* and *B*.

V. Modification of Proteins

Electrophoresis can be useful, also, in the detection and characterization of permanent changes in protein molecules. These can be of various natures. They may be the result of chemical or enzymatic treatment, of changes in the physical environment, or of storage under even mild conditions. In some cases, chemical modifications have been applied very successfully in obtaining an insight into the mechanism of the biological activity of a protein.

A. Chemical Changes

A typical example of small chemical changes induced by enzymatic action that has been studied in detail electrophoretically is the ovalbumin-plakalbumin system (42–49). Here, as shown in Section III, A, a combination of chemical and electrophoretic analyses led to an understanding of the system. Another important case that has been discussed above is Singer's work on the antigen-antibody system (106) in which chemical blocking of particular groups with subsequent electrophoretic analysis led to the identification of an ε-amino group as being essential for the formation of the Ag-Ab bond (Section IV, B). In the case of enzymes, reaction of trypsin with DFP led to a change in its heterogeneity pattern as well as its capacity to bind a specific inhibitor (60, 112), while acetylation resulted in a similar change in the electrophoretic heterogeneity pattern (62), which in normal trypsin is contingent on the binding of some specific ions; in the last case, however, there is no loss of the ovomucoid binding capacity, nor of enzymatic activity (Section III, A). A few other types of studies that have been carried out will be mentioned briefly, while no attempt will be made to cover the subject completely.

Dreyer and Neurath (150) and Pechère and Neurath (151) have used Tiselius electrophoresis in very elegant studies on the rates of activation of chymotrypsinogen and trypsinogen, respectively.

In Fig. 20 are given electrophoretic patterns of the chymotrypsinogen-chymotrypsin transformation system (150). The electrophoretic pattern of chymotrypsinogen indicated that protein to be at least 97% homogeneous. During the activation, as can be seen on Fig. 20, the boundary corresponding to chymotrypsinogen was gradually replaced by components of decreasing mobility identified as π- and δ-chymotrypsin. At intermediate times of activation, the π-enzyme predominates, while at the end of 9 minutes of activation, at least 90% of the protein had a mobility charac-

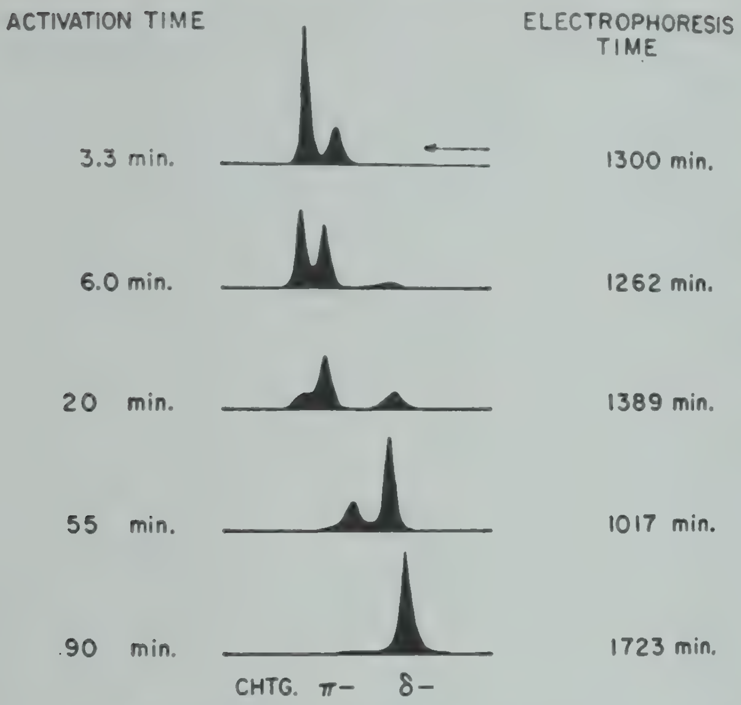

FIG. 20. Ascending electrophoretic patterns of chymotrypsinogen (CHTG.) activation mixtures. Acetate buffer, pH, 4.97, Γ/2, 0.1; 5.8 volts per centimeter. The ascending mobilities are: chymotrypsinogen, 3.8×10^{-5}; π-chymotrypsin, 3.65×10^{-5}; δ-chymotrypsin, 3.25×10^{-5} cm^2-$volt^{-1}$-$sec.^{-1}$ (150).

teristic of δ-chymotrypsin. In this manner, it was possible to follow the kinetics of formation and disappearance of the various components which take part in this transformation.

A similar study was carried out on the trypsinogen-trypsin system (151). In this case, a complication arose due to the fact that the trypsin had to be inhibited as it was formed. Therefore, the research was carried out in the presence of soybean trypsin inhibitor. After accounting properly for the contribution of the inhibitor to the area on the electrophoretic patterns, Pechère and Neurath were able to obtain a curve for the rate of formation of trypsin which agreed quantitatively with data obtained from activity measurements.

Free electrophoresis has been used extensively in the study of the interaction of proteins with detergents. These have been mentioned above (Section IV, A) and reviewed thoroughly by Putnam (98). In all cases studied, the protein was altered to some extent. An interesting example is the work of McMeekin and co-workers (152), who isolated a crystalline complex of β-lactoglobulin with dodecyl sulfate, containing two equivalents of detergent per molecule of β-lactoglobulin (mol. wt. 35,000). Electrophoretic data in pH 4.8 acetate buffer of 0.1 ionic strength resulted in a single slightly skewed peak of reduced mobility, while in pH 8.4 barbital of 0.1 ionic strength the mobility of the complex was more negative than that of the native protein. In that pH region, titration data showed the acid-binding capacity of the complex to be two equivalents lower than that of the native protein. At pH's close to 4, the titration curves were approximately the same. The observation would seem to indicate that binding of the detergent has a strong effect on the association reaction at pH 4.8 (see Fig. 14).

Recently, Foster and Aoki (139) have reinvestigated the interaction of bovine serum albumin with sodium dodecyl sulfate and interpreted the data in terms of the proposed *N-F* isomerization of this protein (see Section IV, E) (134–139). It was found that the detergent has a pronounced effect in displacing the equilibrium toward the *N* form. The dependence of the electrophoretic composition on detergent ion concentration could be fitted most accurately by assuming that the *N* form possessed ten strong binding sites of intrinsic dissociation constant 2.7×10^{-4}. It should be noted that in this interpretation the electrophoretic boundaries are considered to be due to the presence of two forms of the protein rather than to protein and complex, as was found to be the case in earlier studies (98, 100).

Other typical chemical modifications of proteins have been mentioned in previous sections (III and IV). In general, it is found that, outside of a change in mobility, chemical modification of proteins has little effect on their electrophoretic properties. A discussion of this is available (153).

B. Denaturation

Another type of transformation which occurs in proteins is that generally described as denaturation. This covers a broad spectrum of changes which vary in nature over a wide range but usually do not involve the breaking of protein covalent bonds. In general, the information revealed by Tiselius electrophoresis on denatured proteins is not very extensive and usually amounts to changes in mobility and the occasional formation of new moving boundaries. A review of this subject is available (154).

An interesting recent application of electrophoresis to the problem of denaturation can be found in the work of Tsiperovich and Loseva (155) who have studied the denaturation of ovalbumin in the presence of urea. From the results, shown in Fig. 21, the authors concluded that when ovalbumin is denatured in the presence of urea, the process is of an all-or-none type, since as denaturation progresses, the area under the native protein boundaries decreases gradually, while a new boundary attributed to denatured protein increases at the same time. From the data of Tsiperovich

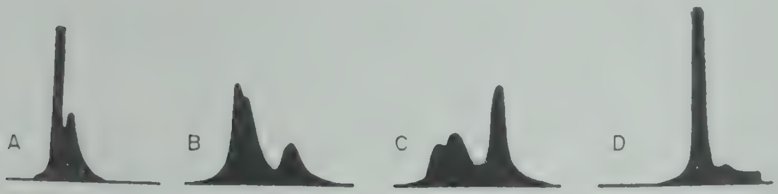

FIG. 21. Electrophoretic diagrams showing progress of ovalbumin denaturation in urea (159). Protein concentration: 2.33%; pH 7.2, 0.05 *M* phosphate buffer; urea concentration: 400 mg/ml; 180 minutes at 2.5 volts/cm. A, native ovalbumin; B, 30% denatured ovalbumin; C, 47.9% denatured ovalbumin; D, 95–100% denatured ovalbumin.

and Loseva it would seem also that the A_1 component is denatured more rapidly than the A_2 component, since the relative area under its peak decreases at a faster rate.

Briggs and Hull (156) have studied the kinetics of denaturation of β-lactoglobulin by Tiselius electrophoresis in a pH 6.9 phosphate buffer of 0.1 ionic strength. By following the change in component areas with time, they were able to conclude that at pH 7 two distinct processes occur in the denaturation of that protein. The first process, which is initiated at temperatures above 65°C., is accompanied by a fourfold increase in particle weight, but little change in electrophoretic properties. The second process, which can take place only after the first one has occurred, proceeds at lower temperatures and is accompanied by a marked increase in electrophoretic mobility and a large increase in particle weight.

Another important related application of electrophoresis is the study of gross composition changes in biological systems on exposure to various conditions. In some cases, rather profound changes in electrophoretic composition may occur even on exposure to mild conditions. In general, permanent changes in proteins under a variety of conditions have been observed by electrophoresis in a large number of systems. A few typical cases might be cited as examples. Longsworth (9) have found that ovalbumin undergoes a transformation on exposure to acid pH's with the formation of a new electrophoretic component. The denaturation of serum proteins on exposure to pH's below 4.0 has been studied electrophoretically by Perlmann and Kaufman (157) with the finding that a new component with a mobility intermediate between those of α_2- and β-globulins is formed. A single peak results from the denaturation of serum proteins with ultraviolet irradiation (158). This has been attributed to the unwinding of the coiled molecules. Tsiperovich and Loseva (159) have reported on the formation of a "stabilized form," with a higher electrophoretic mobility, of ovalbumin upon treatment with urea at pH 7.0. These are far from exhaust-ing the vast literature which exists on similar electrophoretic transformations, but a complete coverage of the field is far beyond the scope of this chapter.

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Chapter 9

Clinical and Physiological Applications of Electrophoresis

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Following the introduction of the improved moving boundary apparatus of Tiselius in 1937, applications of electrophoresis to clinical and physiological studies have been made in many branches of the biological sciences. The applications have been most numerous in the clinical field, but most profitable in biochemistry. Wherever it has been possible to obtain biological substances in an aqueous solution the method has been applied. The literature on the subject is now so abundant that the task of writing an entire volume might be easier than writing a single chapter. Nevertheless, many reports, particularly those of a clinical nature, do not lend themselves to correlative interpretation. For example, much of the literature dealing with pathological plasma contains data indicating nonspecific variations from the normal. This chapter, therefore, will deal only with the more fundamental and interpretable aspects of the clinical and physiological applications of electrophoresis, without attempting to review the total literature.

I. PLASMA

A. *Nature and Origin*

Electrophoresis provides an excellent method for fractionating protein mixtures without undue denaturation. The method has been applied most extensively to the study of blood plasma, where there has been an attempt to correlate the plasma pattern with states of health, disease, and stress. In order to acquire a basis for interpreting these analyses and to assign real significance to pattern variation, it is necessary to have some understanding of the nature and origin of the plasma components. Accordingly, the first section of this chapter will be devoted to providing a background for understanding and interpreting plasma patterns.

Histologically blood is considered to be one of the four types of connective tissues. The embryonic mesenchyme develops from the mesoderm immediately after the formation of the germ layers, and the connective tissue rapidly amasses between the layers. In the earliest stages of development the endothelial cells of the blood vessels and the blood cells arise simultaneously from the same mesenchymal elements. These embryonic cells undergo many changes to form the various blood and connective tissue cells. In adult vertebrates 7–8% of the body weight is blood and a little more than half of the blood is plasma (50–60% by volume). The concentration of protein in the plasma varies from 6.5 to 8.5 gm. per 100 ml. The distribution of the ions that contribute to the total ionic strength of plasma is indicated by the chart of Fig. 1 (1). Although the proteins contribute little to the total ionic strength, owing to their large molecular size, the mass is some twenty times greater than the total sodium. Over 85% of the total plasma solids is protein.

Among other constituents of plasma are the lipids which include fats, fatty acids, lecithin, and cholesterol. Glucose is the only free carbohydrate in normal serum. In this discussion of plasma we shall be concerned primarily with the complex nature and significance of the proteins, the bound lipids, and the bound carbohydrates.

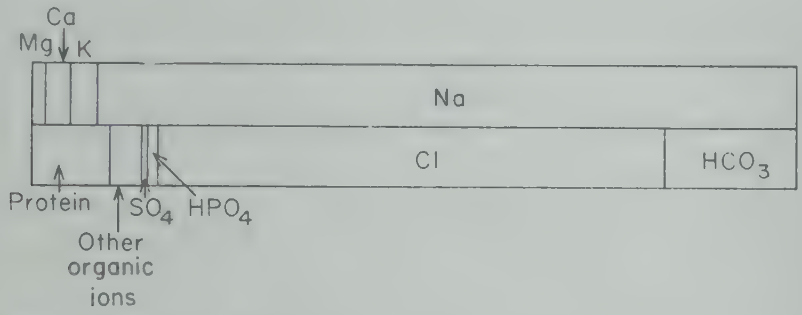

FIG. 1. Chart indicating the relative number and kind of ions in plasma. (1)

Prior to the middle of the last century, blood physiopathology was based primarily on the red cell, anemia being the principal concern and the only recognized pathology. Later the importance of the leucocytes was recognized and they were intensively studied; but it was not until the third and fourth decades of the present century that techniques had been developed to the extent that useful information could be obtained from a study of the plasma proteins. A series of important developments during these decades contributed to the advancement in knowledge of these elements. Improvements were made in methods of separating and characterizing the various constituents of plasma, fractionations being accomplished by the salt-precipitation method of Howe (1921), by ultracentrifugation (Svedberg, 1927), by electrophoresis (Tiselius, 1937), and by cold alcohol precipitation (Cohn, 1939). The number of studies of normal, experimentally altered, and pathological plasma and serum utilizing these methods has now become enormous.

The plasma proteins are made up of a complex mixture of components which differ in properties and function. The major components of the plasma proteins include: (a) the albumins, (b) the various globulins, (c) the fibrinogen, (d) the lipoproteins, nucleoproteins, and seromucoids, which may also be classified as globulins or albumins.

By means of electrophoresis and ultracentrifugation, more than twenty major fractions of human serum have been quantitated. A three-dimensional display of the major nonlipid fractions is reproduced in Fig. 2 (2). The lipoproteins were removed by centrifugation. The quantity of each component varies somewhat from one serum to another, but the display gives a visual concept of their relative magnitudes. The immunoelectrophoretic procedures of Grabar and co-workers (3, 4) have also demon-

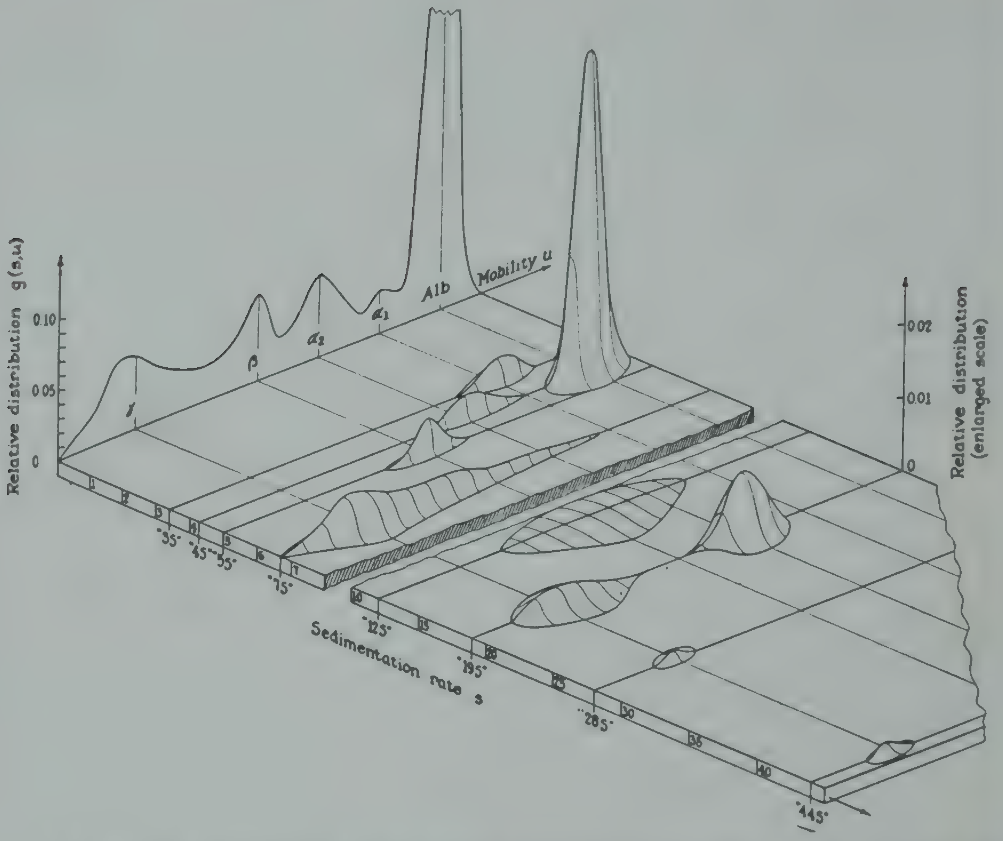

FIG. 2. Three-dimensional display showing electrophoretic and ultracentrifugal components of lipid-free normal human serum (2). The sedimentation rate and relative mobility of each component are indicated. Gamma globulin contains four different molecular species.

strated the very large number of antigenic entities which exist in serum.

The study of plasma fractions has been greatly extended by the use of cold alcohol precipitation procedures developed by Cohn and collaborators [for details of this procedure see Cohn, *et al.* (5)]. The electrophoretic distribution of the principal cold alcohol-precipitated fractions is given in Table I (6).

The lengths and diameters given in the last column of this table have been calculated from the molecular weights, the partial specific volumes, and the sedimentation constants. Birefringence of flow and viscosity measurements of solutions containing the different plasma fractions furnish corroborative evidence for the aspherical shape of most of the molecules. The resolution now obtainable in the electron microscope is adequate to visualize these macromolecules directly if suitable preparations could be made. At a resolution of 10–20 Å and a magnification of 10^6, a very thin section of plasma as it would appear in the blood is illustrated in Fig. 3. Attempts to visualize plasma components have not yet been very successful

TABLE I

PROTEIN COMPONENTS OF NORMAL HUMAN PLASMA CHARACTERIZED BY PHYSICAL CHEMICAL METHODS[a]

Electrophoretic Component	Fraction	Approximate amount in plasma (gm./l.)	Sedimentation Constant $S_{20,w}$	Specific Volume V	Intrinsic Viscosity $H_0 \times 10^2$	Frictional Ratio f/f_0	Molecular Weight M	Approximate Dimensions (Ångströms) Length	Approximate Dimensions (Ångströms) Diameter
Albumin	V	32	4.6	0.733	4.2	1.28	69,000	150	38
α_1-globulin	IV–1	2[b]	5.0	.841	6.6	1.38	200,000	300	50
α_2-globulin	IV–6	1	9.	.693	9.2	1.58	(300,000)	—	—
β_1-globulin	IV–7	2	5.5	.725	5.5	1.37	90,000	190	37
β_1-globulin	III–0, III–2	2	7.	.74	—	—	(150,000)	—	—
β_1-globulin	III–0	1	20.	.74	—	—	500,000 1,000,000	—	—
β_1-globulin	III–0	2[b]	2.9[c]	.950	4.1	1.7[c]	1,300,000	185	185
β_2-globulin	III–1	2	7.	—	—	—	(150,000)	—	—
γ-globulin	II	5	7.2	.739	6.	1.38	156,000	235	44
γ-globulin	II	1	10.	.739	—	—	(300,000)	—	—
Fibrinogen	I–2	2	9.	—	25.	1.98	400,000	700	38

[a] From Oncley *et al.* (6).

[b] These two globulins are lipoproteins containing 35% lipid for the α_1-globulin and 75% lipid for the β_1-globulin. The other components contain little or no lipid.

[c] This is the sedimentation constant obtained in 0.5 M sodium chloride solution, when corrected in the usual manner. The f/f_0 value given here is the value for sedimentation, correcting for the partial specific volume of the hydrated protein (0.97), using the equation of Kraemer (equation 124, p. 65, reference 7). A solvation of 0.6 g. of water per g. of protein was used for this calculation, and the molecule was assumed spherical.

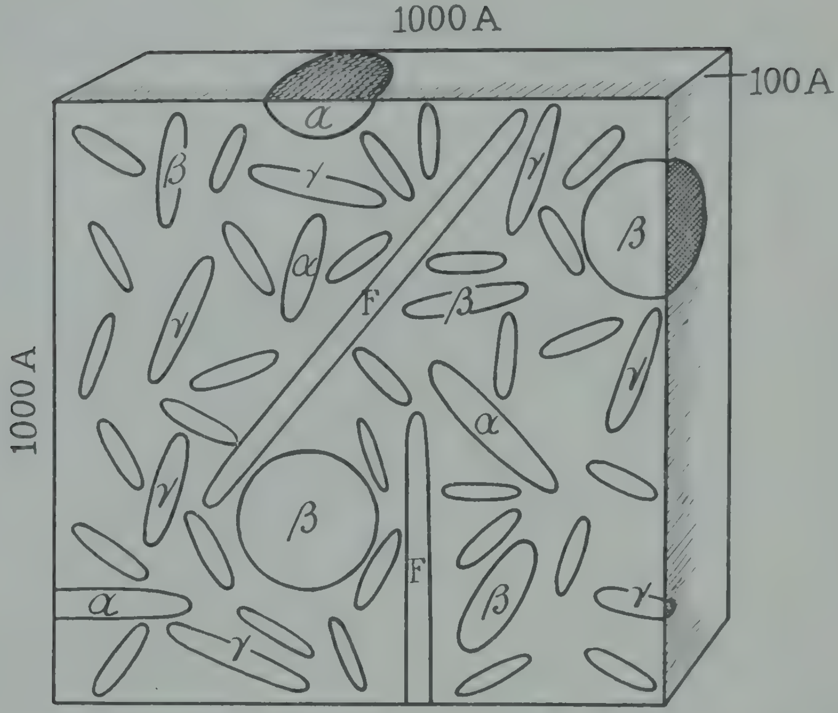

FIG. 3. A schematic section of plasma at a magnification of one million. The plasma protein molecules are drawn from information on morphology and concentration presented in Table I. In electron microscopy, sections 2 to 5 times thicker than the one indicated here are used in actual practice and would therefore contain a greater number of molecules. Moreover the molecules would be oriented at random in the third dimension. For the sake of clarity the particles found in the indicated volume are all drawn with their long axes in the plane of the paper.

The molecules are labeled as follows: Unlabeled = albumin, Fraction V (Cohn); α = α-globulin, Fraction IV; β = β-globulin, Fraction III; γ = γ-globulin, Fraction II; F = fibrinogen, Fraction I.

There are many other molecular species present, as is indicated in Figs. 1 and 7, but their frequencies are so low that they would not likely appear in the small section illustrated. Glucose molecules are about 10 times, and sodium ions about 240 times, more frequent than albumin molecules.

due to the difficulties of fixing, dehydrating, and observing with the electron beam without destroying the structure. The data of Table I indicate that the diameter of most of the molecules falls within the narrow range of 37 to 50 Å although the lengths vary greatly. Electron micrographs of fibrinogen and γ-globulin preparations reveal mostly spherical particles with diameters of 30-40 Å (7, 8). Often longer, beaded particles are formed by a linear arrangement of three or more of the spherical or near spherical elements. The molecular weight of 30 Å spheres would be about 11,000 and that for 40 Å spheres would be about 24,000. The early studies with the ultracentrifuge led Pedersen (9) to postulate that proteins were formed from basic units having a molecular weight of about 17,000. The general

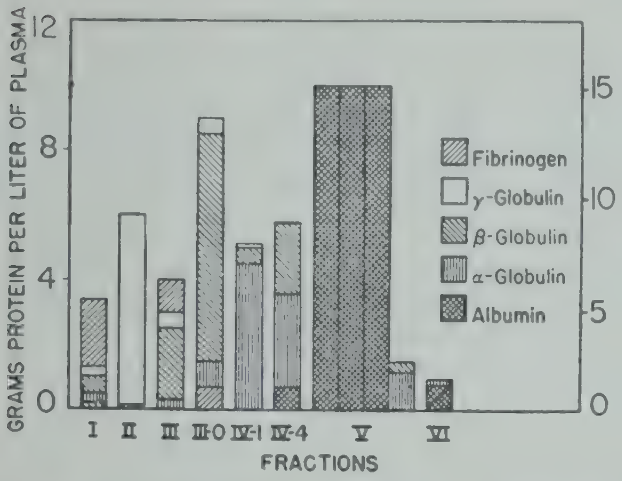

FIG. 4. Distribution of electrophoretic components in fractions of human plasma separated by cold alcohol precipitation. Fraction V is so large that it occupies more than three columns (6).

idea of basic units being linked together by secondary forces is not inconsistent with what is seen in the electron microscope. If the basic units varied in size and composition, the resulting protein molecules or molecular compounds (10) could easily have the wide range of particle weights that are actually found. Furthermore, many substances may be adsorbed on the native protein without having anything to do with its basic structure, but which do affect the size as determined by physical procedures.

Components characterized either chemically or by ultracentrifugation do not have an exact correspondence with electrophoretic fractions, as is illustrated in the charts of Figs. 4 and 5.

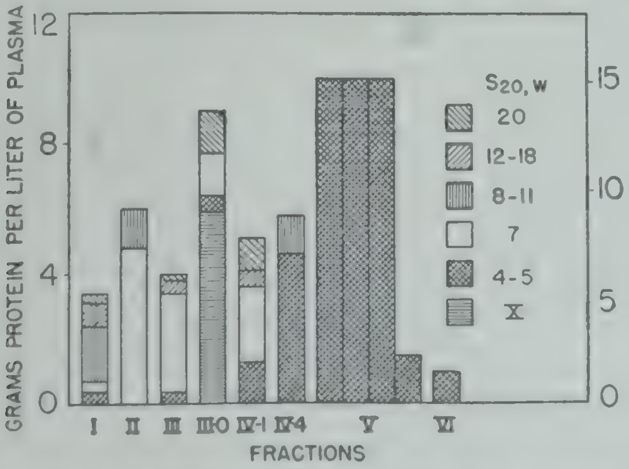

FIG. 5. Distribution of ultracentrifugal components in fractions of human serum separated by cold alcohol precipitation. The sedimentation constant values, $S_{20,w}$ are corrected to 20° C. and a solvent of water. The X component is rich in lipids (6).

1. Albumin

In normal human serum about 60% of the total protein is albumin. Its molecular weight is 66,000 (10a), the smallest of the principal proteins, and its concentration is approximately 6×10^{-4} moles or more than 3×10^{17} molecules per milliliter. Because of its relatively high molecular concentration, it contributes far more to the colloid osmotic pressure of the blood than all of the other proteins combined. A primary function of the albumin is apparently that of maintaining the fluid balance.

It also has vehicular functions with specific powers of binding many substances in the blood. These include both anions and cations of many dyes, the anions of fatty acids and of many aromatic carboxylic acids and acetylated amino acids, many penicillin and sulfonamide derivatives, heparin, and many other compounds. Albumin shows a much stronger tendency to bind these substances than do the other proteins of plasma, although the albumin can be saturated, in which case the excess of added material may be bound to some extent by globulin or may remain free in the plasma (10b). This binding ability of the serum albumin has far-reaching biological significance, as was first emphasized by Bennhold (11, 12). It is able to bind and inactivate toxins and other metabolic inhibitors (13).

Associated also with the albumin fraction are certain enzyme inhibitors. For example, the action of bovine testicular hyaluronidase on human umbilical cord hyaluronic acid is inhibited by electrophoretically separated human serum albumin, but not by the other serum components (14, 15). Plasminogen (16) and other enzymes may be separated from their inhibitors by proper fractionation of the serum.

a. Synthesis of albumin. The liver seems to be the organ primarily responsible for the synthesis of albumin. In patients with severe liver diseases such as cirrhosis, Banti's syndrome, and chronic hepatitis (17), there is a marked decrease in the amount of circulating serum albumin. That albumin is present within hepatic cells is indicated by the fact that a protein with the mobility of albumin may be found in liver extracts after the tissue has been perfused to remove the blood of the vascular bed (18). Extraction with a 1:1 mixture of 2% NaCl and butyl alcohol yields eight components with the one having the mobility of albumin representing 10-15% of the total. By incubating chicken liver slices in a bicarbonate medium, Peters and Anfinsen (19) showed that the amount of serum albumin both in the medium and in homogenates of the slices increased during the period of incubation. When the slices were incubated in the presence of radioactive bicarbonate or radioactive glycine the albumin became radioactive. Similarly, by perfusing whole rat livers with whole aerated blood, Miller *et al.*

(20) were able to maintain apparently normal metabolism for many hours and concluded that the liver synthesizes practically all of the fibrinogen, he albumin, and more than 80% of the globulin. Their experiments consisted of adding C^{14}-labeled amino acids to the mechanically circulated, heparinized and oxalated blood and measuring the rate of their incorporation into the plasma proteins. Samples of blood were taken at hourly intervals after the addition of amino acids; the plasma protein was precipitated by trichloroacetic acid and fractionated by Majoor's method (21). By means of their perfusion technique, it was shown that the percentage of amino acids converted by the liver to CO_2 and the percentage utilized in liver and plasma protein synthesis was the same as in the intact animal. They further showed that the liver was able to discriminate between *d*-lysine and the natural form, *l*-lysine. Campbell and Stone (22) also incubated liver tissue slices with C^{14}-glycine and obtained an incorporation of radioactivity into the soluble tissue proteins precipitated by serum albumin antiserum. Radioactive amino acids were found in both the soluble tissue proteins and the serum albumin of such liver slices. A net synthesis of serum albumin did *not* occur, however, when rat *kidney* slices were incubated, nor did the serum albumin contained in such slices become radioactive when they were incubated with C^{14}-glycine, although the soluble tissue proteins did become radioactive in the kidney slices. These workers also studied liver tumors induced by feeding 4-dimethylaminoazobenzene and demonstrated that the hepatoma cells, but not the cholangioma cells, were capable of synthesizing serum albumin.

There seems, consequently, to be little doubt that liver is responsible for the synthesis of serum albumin as well as fibrinogen, and it is likely that portions of α- and β-globulins are also synthesized in the liver.

b. The complexity of serum albumin. Although serum albumin appears electrophoretically homogeneous at neutral or slightly alkaline pH values, variation in conditions of analysis have demonstrated its complexity. Two electrophoretic components were found in crystalline horse serum albumin which had constant solubility and was homogeneous in the ultracentrifuge. The components were detected upon electrophoretic analysis in acetate buffer at pH 4.0, ionic strength 0.02 (23). The mobilities of the two components were 6.8 and 6.4 × 10^{-5} cm²/volt-sec., respectively, about 60% of the protein moving at the faster, and 40% at the slower, rate. When the same protein was re-examined, however, at pH 7.4 it migrated with a single boundary.

Normal human serum albumin studied under the same conditions had two components with mobilities of 7.2 and 5.9 × 10^{-5} cm²/volt-sec., respectively. The resolution of the two components of the human was, therefore, appreciably greater than that for the horse serum albumin. In human serum albumin the component with the higher mobility was about twice as large as the slower-migrating component (Fig. 6). In certain diseased states, the individual components vary. In terminal nephritis only the faster-moving fraction was slightly reduced; in nephrosis the faster fraction was reduced to one-thirtieth its normal value and the slower fraction was reduced to one-sixth normal; in cirrhosis the slower fraction was unchanged but the faster fraction was reduced to one-third its normal value. Thus, in the nephrotic syndrome and in cirrhosis the ratio of components was reversed from the normal. This reversal appeared in both urine and blood, however, and therefore the abnormality cannot be attributed to selective urinary excretion but must be due to differences in protein production (23). Two crystalline albumins with different mobilities have been isolated from horse serum by McMeekin (24). The slower-moving component contained carbohydrate, whereas the faster-moving one did not.

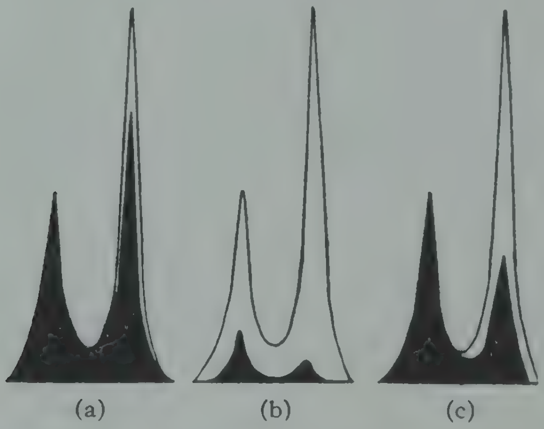

FIG. 6. Electrophoretic diagrams of pathological human albumins (black) compared with the normal (line tracings) analyzed at pH 4.0; (a) terminal nephritis, (b) nephrosis, (c) cirrhosis (23).

Two albumin components were found in the serum of young rats by Halliday and Kekwick (25), who used a phosphate buffer at pH 8.0 (see Section IB2). A small component moving ahead of albumin was also reported in the plasmas of several species by Deutsch and Goodloe (26).

Recrystallized horse serum albumin, which migrates as a single component in phosphate buffer at pH 7.4, has a tendency to change slowly into a component with lower mobility (27). From sedimentation and diffusion measurements the molecules of the slower-migrating component were found to be almost one and one-half times larger than those of fresh albumin. The second molecules probably result from a rearrangement of elements of the first.

2. *Alpha and Beta Globulins*

When Tiselius first studied human serum in his new apparatus, the conditions were such that only four components appeared. It was established that one in highest concentration, which also had the highest mobility, corresponded roughly to chemically separated albumin. The other three fractions were named arbitrarily α-, β-, and γ-globulin in order of decreasing mobility.

The number of electrophoretic fractions can be varied by varying the conditions of study, and the three globulins of serum originally described by Tiselius have turned out to be more complex. In 1942 Longsworth (28) reported that when a different buffer was used at a higher pH, another component in human serum which had previously moved with albumin then moved with a mobility between that of albumin and α-globulin. This new component was called α_1-globulin and the veronal buffer, pH 8.6, described by Longsworth has since been used by most investigators who

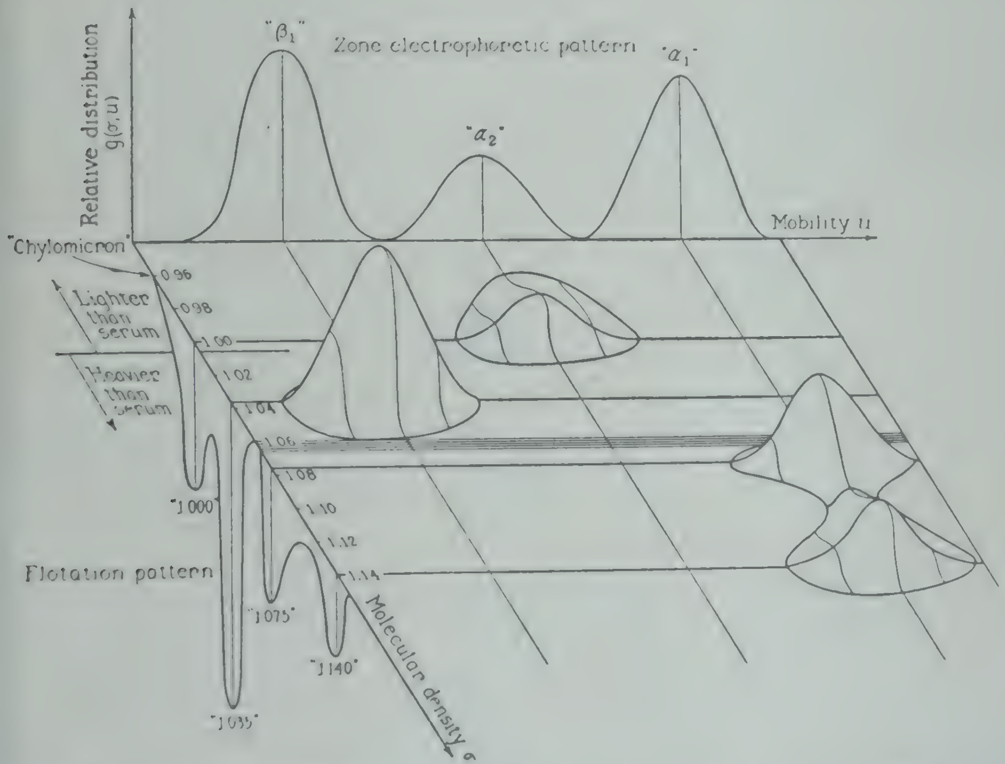

FIG. 7. Three-dimensional display of the electrophoretic and ultracentrifugal components of lipoproteins of human serum. The chart is somewhat idealized, because the distribution of lipoproteins differs considerably with the type of measure used; here phospholipid is implied for both the mobility dimension and for zones successively floated out of the serum by increasing the solvent density (30).

have studied human serum. The IV–1 fraction of Cohn contains a lipid-rich component, the α_1-lipoprotein, which when redissolved has a mobility similar to native α_1-globulin (29). The general distribution of lipoproteins in human serum can be visualized by the three-dimensional chart of Kunkel and Trautman (30) reproduced in Fig. 7. The data were obtained from ultracentrifugal studies, in solvents of different densities, of electrophoretic fractions isolated from starch or polyvinyl resin columns. The inverted pattern along the diagonal axis corresponds approximately to the analytical flotation pattern at a solvent density of 1.2. There are two α_1-lipoproteins with molecular densities of approximately 1.075 and 1.140 and one α_1- and one β_1-lipoprotein with densities 1.000 and 1.035, respectively. This pattern is somewhat idealized and may not hold for all normal human sera. Pathological sera will show at least quantitative variations. Manipulations may also cause variations in distribution of lipoproteins.

The main component of a mixture of mucoproteins, isolated from perchloric acid (0.6 *M*) filtrates of human plasma by saturating with ammonium sulfate at pH 4, has a mobility of α_1-globulin (31). Patterns of four different preparations made by this method and one prepared entirely by sodium sulfate fractionation are reproduced in Fig. 8. These mucoproteins (15–35% carbohydrate) constitute the proteose of human serum. They contain hexosamine and sulfur and are low in nitrogen content in comparison to other proteins. Their concentration in normal plasma expressed in

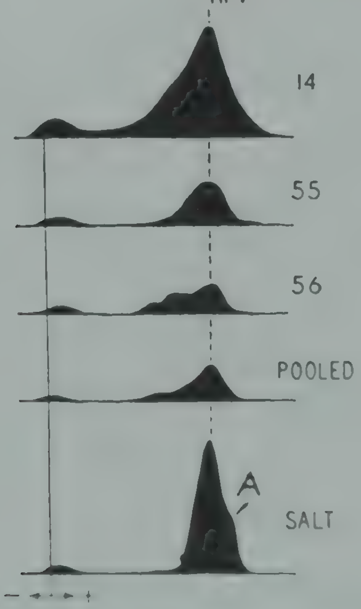

FIG. 8. Electrophoretic patterns of different preparations of a mucoprotein, MP1. The principal component migrates as α-globulin. The "salt" preparation contained 31% albumin, which may be seen as a shoulder in the pattern (31).

terms of carbohydrate is about 15 mg. per cent. The distribution of hexosamine in the fractions of normal human serum has been measured by Bollet *et al.* (32). The amount in albumin was too small for accurate determination, but mean values in milligrams per cent for the globulins were as follows: α_1-globulin, 17.5; α_2-globulin, 25.0; β-globulin, 17.4; γ-globulin, 24.7. In rheumatoid arthritis and pneumonia the hexosamine content of each component was found to be elevated and in the α_1-globulin the ratio of hexosamine to protein was almost doubled in these diseases. In cirrhosis the hexosamine of the γ-globulin was markedly elevated, as was also the protein. The significance of these carbohydrate fluctuations is not yet understood. Glycoproteins (all protein-bound polysaccharides) have been shown to be distributed in all of the plasma fractions of normal human serum, as illustrated in Fig. 9 (33). The distribution of the total glycoproteins varies greatly in a number of diseases, such as idiopathic thrombocytopenic purpura, collagen diseases, and lupus erethematosus, often being associated also with the γ_2-globulin.

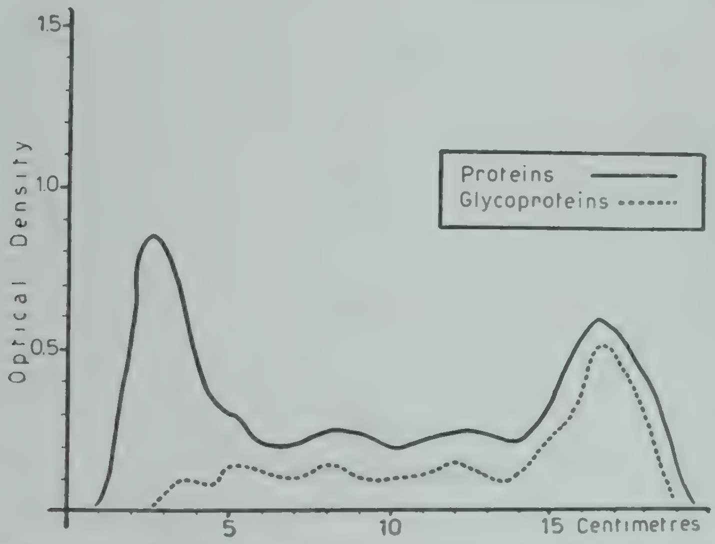

FIG. 9. Electrophoretic distribution of serum proteins and glycoproteins in normal subjects (semischematic from paper electrophoresis) (33).

Properdin (34) is a normal constituent of β-globulin (Cohn Fraction III). This globulin with a molecular weight of more than one million participates in such diverse activities as the destruction of bacteria and neutralization of viruses and in the hemolysis of red cells under certain conditions. It acts only in conjunction with complement and magnesium ions. The concentration of properdin is low, less than 2 mg. per milliliter of serum, and varies greatly from one species to another, being high in the rat, intermediate in man, and low in the guinea pig. A correlation of these levels with natural resistance to infection has been made (34).

The complex α- and β-globulins serve in the transport and exchange of fats and fat-soluble materials. The large quantities of cholesterol, phospholipids, fatty acids, and the various fat-soluble vitamins and hormones are for the most part not free in the plasma, but are transported by these globulins, which hold them in solution in relatively high concentration although they are virtually insoluble in pure water or aqueous salt solutions. They are composed of molecules varying in molecular weight from 90,000 or less to more than a million. The number and magnitude of electrophoretic components in human serum falling within the group of α- and β-globulins vary widely both in normal and in the abnormal or pathological states. Different species show still wider variations in these fractions. The site of synthesis of all of these serum fractions is not yet known. Undoubtedly part of it takes place in the liver (20), but other organs and cell types seem to be capable of synthesizing at least certain components of the α- and β-globulins. (See discussion of synthesis in the human placenta (pages 401–402) and also in relation to seminal plasma (Section II, F.)

It is an interesting observation that the known plasma protein-synthesizing cells have direct contact with the blood without intervening capillary endothelia and their basement membranes. From studies of liver with the electron microscope there appears to be no endothelial barrier between the parenchymal cells and the blood (35), and it would seem unlikely that proteins are transported through cells except by the mechanism of cytopempsis because, if free in the cytoplasm, they would undergo alterations. In cytopempsis the transmitted material is enclosed and protected by cytoplasmic membrane (36). However, the transport of materials through capillary walls is not yet fully understood.

3. Gamma Globulins

The γ-globulins have the highest isoelectric range of any of the major plasma proteins. They are composed primarily of nonspherical (see Fig. 3) molecules having a molecular weight of approximately 160,000, although small amounts of larger molecules have been demonstrated. Human γ-globulin contains 10–15% of a component having a molecular weight of about 1,000,000 as well as small amounts of other molecular species as is illustrated in Fig. 2. The heavy component (19S) migrates in the forepart of the γ-globulin group (γ_1-globulin) and is relatively rich in carbohydrates, containing hexose, fucose, hexosamine, and sialic acid (37). Most antibodies are contained in the γ-globulins, although antibody activities have been found in almost all the other plasma fractions. The distribution of antibodies will be discussed later. Two distinct fractions, termed γ_1- and γ_2, have been separated from the serum of several species by cold alcohol or salt precipitations (38). The isoelectric points and the conditions under which they were determined are given in Table II. Alberty and his associates have further shown that some of these fractions are also heterogeneous, as demonstrated by reversible boundary spreading (39). By immunoelectrophoretic analyses Williams and Grabar (40) have also demonstrated the inhomogeneity of γ-globulin. Gamma globulin, therefore, cannot be said to have an isoelectric point but rather a wide isoelectric range.

TABLE II

ISOELECTRIC POINTS OF GAMMA GLOBULINS[a]

	Average Isoelectric Points		
Protein	Isoelectric pH	Ionic Strength	Buffer
Human γ_1, normal	5.8	0.10	NaCac, NaOAc, NaCl[b]
	6.6	0.01	NaCac
Human γ_2, normal	7.3	0.10	NaCac, NaCl[b]
	8.2	0.01	NaV
Horse γ_1, hyperimmune	5.6	0.10	NaCac, NaOAc, NaCl[b]
Horse γ_2, hyperimmune	7.6	0.10	NaV, NaCl[b]
Cow γ_1, hyperimmune	5.9	0.10	NaCac, NaOAc, NaCl[b]
	6.4	0.01	NaCac
Cow γ_2, hyperimmune	7.3	0.10	NaV, NaCl[b]
	7.5	0.01	NaV, NaCac
Horse, antipneumococcus[c]	4.4	0.17	NaCl, NaAc[d]
Cow, antipneumococcus[c]	4.8	0.17	NaCl, NaAc[d]
Pig, antipneumococcus[c]	5.1	0.17	NaCl, NaAc[d]
Rabbit, antipneumococcus[c]	5.8	0.17	NaCl, NaAc[d]
Rabbit, normal[e]	5.8	0.17	NaCl, NaAc[d]

[a] From Alberty (38).

[b] Sodium chloride makes up 80% of ionic strength.

[c] Salt-dissociated specific precipitate.

[d] Sodium chloride makes up 88% of ionic strength.

[e] Isolated electrophoretically.

a. Synthesis of γ-globulin. Many experiments have been performed in attempts to determine the source of γ-globulin, but only a few will be discussed here. Experiments dealing with the cellular origin of antibodies, which as stated above are at least largely in the γ-globulin fractions, ap-

TABLE III

ELECTROPHORETIC DATA ON LYMPH AND LYMPHOCYTE EXTRACTS FROM NORMAL AND ANTIGENICALLY STIMULATED RABBITS[a]

Specimen		Component								Average weight per node (mg.)	Nodes per 3 cc. pool	Agglutinin titer[b]
		Fast	1	2	3	4	5	6	Total			
Lymph, normal	Composition[c]		200	15	20	10	10	35	290			
	%[d]		69	6.9	6.9	3.4	3.4	12.1				
	Mobilities[e]		5.1	4.4	3.2	2.8	1.6	0.5				
Lymph after stimulation	Composition		705	40	85	20	50	90	990			768
	%		71.2	4.0	8.6	2.0	5.1	9.1				
	Mobilities		4.7	3.4	2.9	2.7	1.5	0.3				
Lymphocyte Extract 1, normal	Composition		75		260			85	420	105	10	
	%		18		62			20				
	Mobilities		5.0		3.4			0.5				
Lymphocyte Extract 2, normal	Composition		75		255			70	400	156	8	
	%		19		63			18				
	Mobilities		5.0		3.6			0.5				
Lymphocyte Extract 3, normal	Composition		20		55			50	125	85	16	
	%		16		44			40				
	Mobilities		4.4		3.4			0.6				
Lymphocyte Extract 1, after stimulation	Composition	40	75		290			115	520	684	6	1024
	%	8	14		56			22				
	Mobilities	6.8	5.1		3.5			0.5				
Lymphocyte Extract 2, after stimulation	Composition	85	100		280			75	545	716	6	2048
	%	16	18		52			14				
	Mobilities	6.3	4.7		3.4			0.5				
Lymphocyte Extract 3, after stimulation	Composition	70	45		165			60	340	730	6	2048
	%	21	13		48			18				
	Mobilities	5.6	4.3		3.5			0.1				
Normal serum	%		70	6		12		12				
	Mobilities		5.4	4.2		3.0		0.9				

peared at one time to indicate that lymphocytes were the responsible cells and might be concerned with their formation (41–44). Other data, however, cast doubt on this hypothesis.

In the first place saline extracts of lymphocytes obtained from antigenically stimulated popliteal regional lymph nodes did not contain appreciable amounts of γ-globulin as determined by electrophoresis and the ultracentrifuge, although the total amount of lymph, protein, and lymphocytes was greatly increased over the normal (45). A component with the mobility of albumin was increased most. Electrophoretic patterns of normal and stimulated lymphocyte extracts are shown in Fig. 10. Data derived from these measurements and those on lymph are given in Table III. The principal difference in the lymphocyte extracts is that the stimulated lymphocyte extracts contain a fast-moving component that is not present in normal extracts. Although there was considerable variation from one preparation to another, in no case was the γ-globulin elevated by the stimulating antigen. Furthermore, the electrophoretically separated slow component did not have a sedimentation constant in the ultracentrifuge similar to γ-globulin; it sedimented much more slowly.

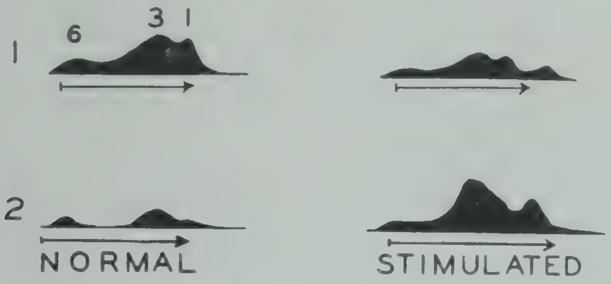

FIG. 10. Electrophoretic patterns of popliteal lymphocyte extract from normal and antigenically stimulated rabbits. A phosphate-saline buffer at pH 7.4, ionic strength 0.2, was used. Sets 1 and 2 show preparation variations. Mobilities are given in Table III (45).

Fagraeus has shown that during the secondary response to intravenous injections of an antigen, especially of living bacteria, there was, concomitant with the increase in circulating antibody, a great increase in the

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[a] From Harris *et al.* (45).

[b] The agglutinin titer is given as an indicator of the concentration of the antibodies to one of the antigens of the dysentery organism. No measure of the total antibodies in the cell extract was available because of the nature of the antigen.

[c] Composition in planimeter units.

[d] Per cent of total area.

[e] The mobilities are given in units of 10^{-5} sq. cm. per volt-second. All components are anodic.

number of plasma cells in the spleen (46). The antigen accumulated in places where plasma cells subsequently developed. Furthermore, pieces of spleen excised at different times during antibody formation and culture *in vitro* revealed that the capacity of red pulp, which contains an abundance of plasma cells, to produce antibodies was considerably superior to that of lymph follicles, rich in lymphocytes but devoid of plasma cells. The highest production of antibodies was at the time when immature plasma cells were numerous but production receded when the culture was predominantly mature cells; however, this difference may not prevail under all conditions.

In a case of plasmacytoma of the mandible in man, where the tumor was composed almost entirely of plasma cells, a saline extract yielded a pattern which was predominantly γ-globulin (Fig. 11). The serum also demonstrated an elevated γ-globulin which returned to normal after removal of the portion of the mandible containing the tumor (47).

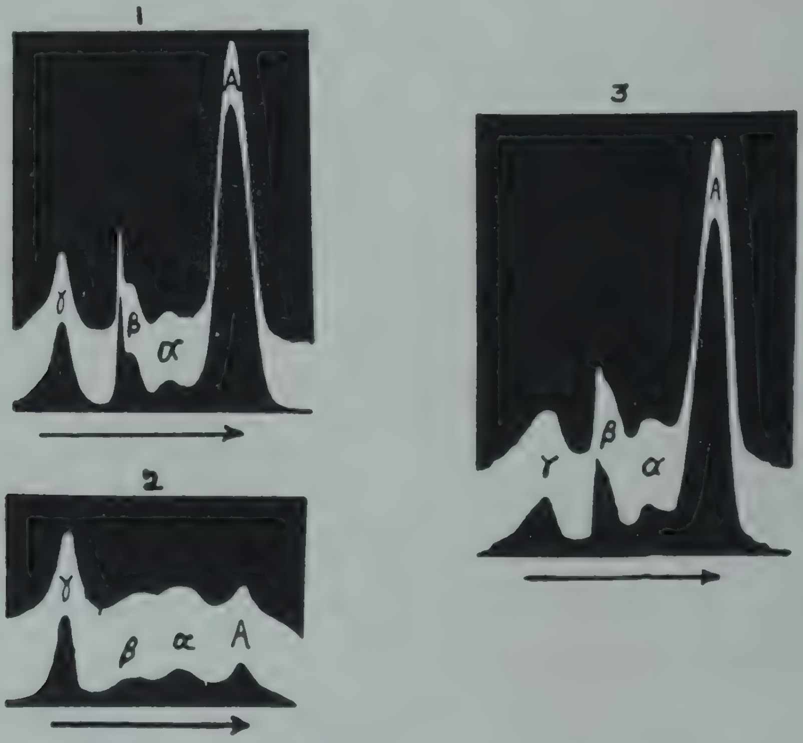


FIG. 11. Electrophoretic patterns of preoperative (1) and postoperative (3) serum and tumor tissue extract (2) from a patient with plasma cell cytoma of the mandible (47).

Thin sections of reticulo-endothelial cells studied in the electron microscope indicate that the cytoplasm of plasma cells is filled with endoplasmic reticulum whose walls are covered with ribonucleic acid (RNA) granules

Palade), making it a likely site for protein synthesis, whereas the lymphocytes contain relatively little endoplasmic reticulum. Electron micrographs of the two kinds of cells are shown in Figs. 12 and 13.

In spite of this accumulation of circumstantial evidence, there is no

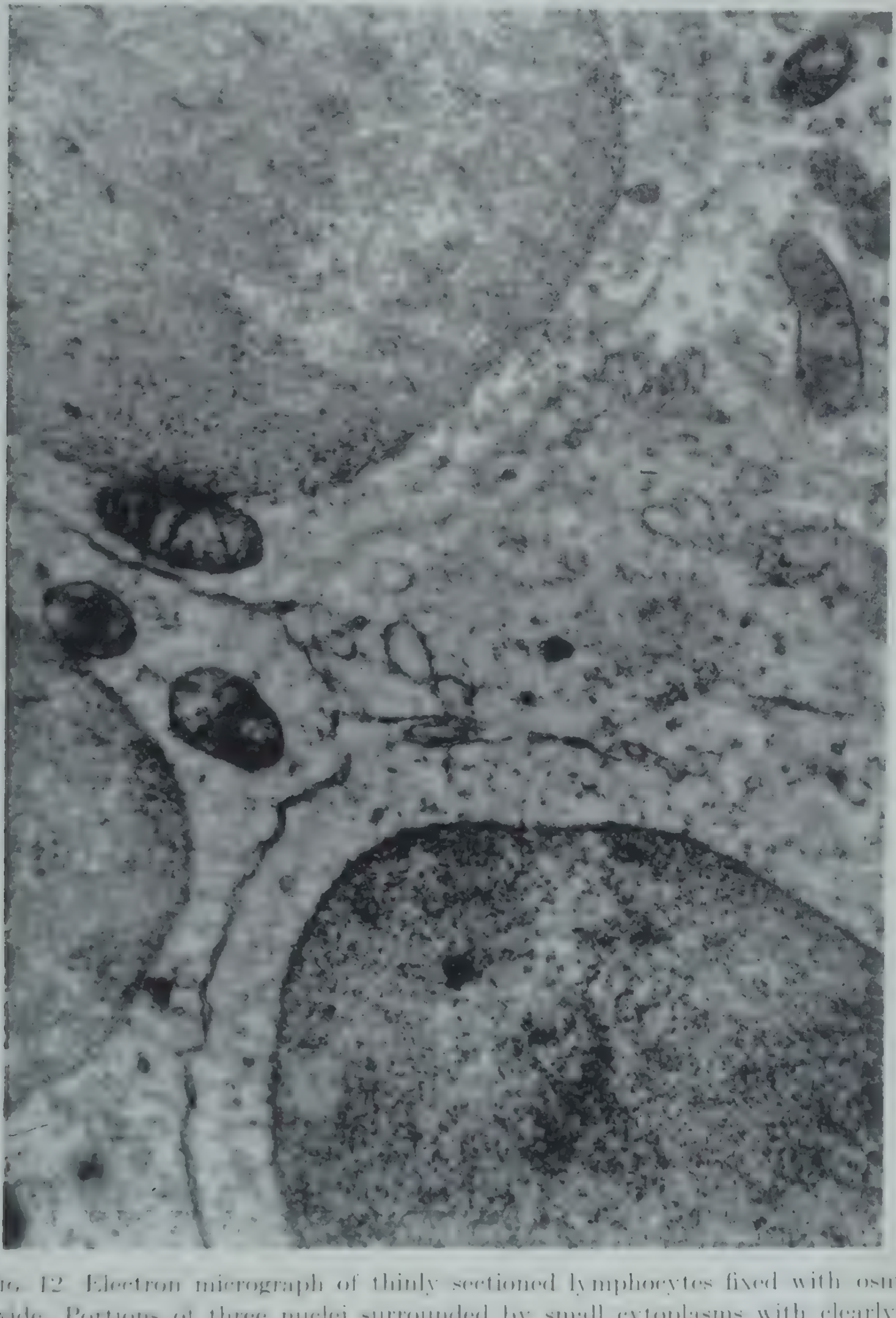


FIG. 12. Electron micrograph of thinly sectioned lymphocytes fixed with osmium etroxide. Portions of three nuclei surrounded by small cytoplasms with clearly demarcated membranes are visible. These cells contain very little endoplasmic reticulum with RNA granules. Magnification: × 35,000.

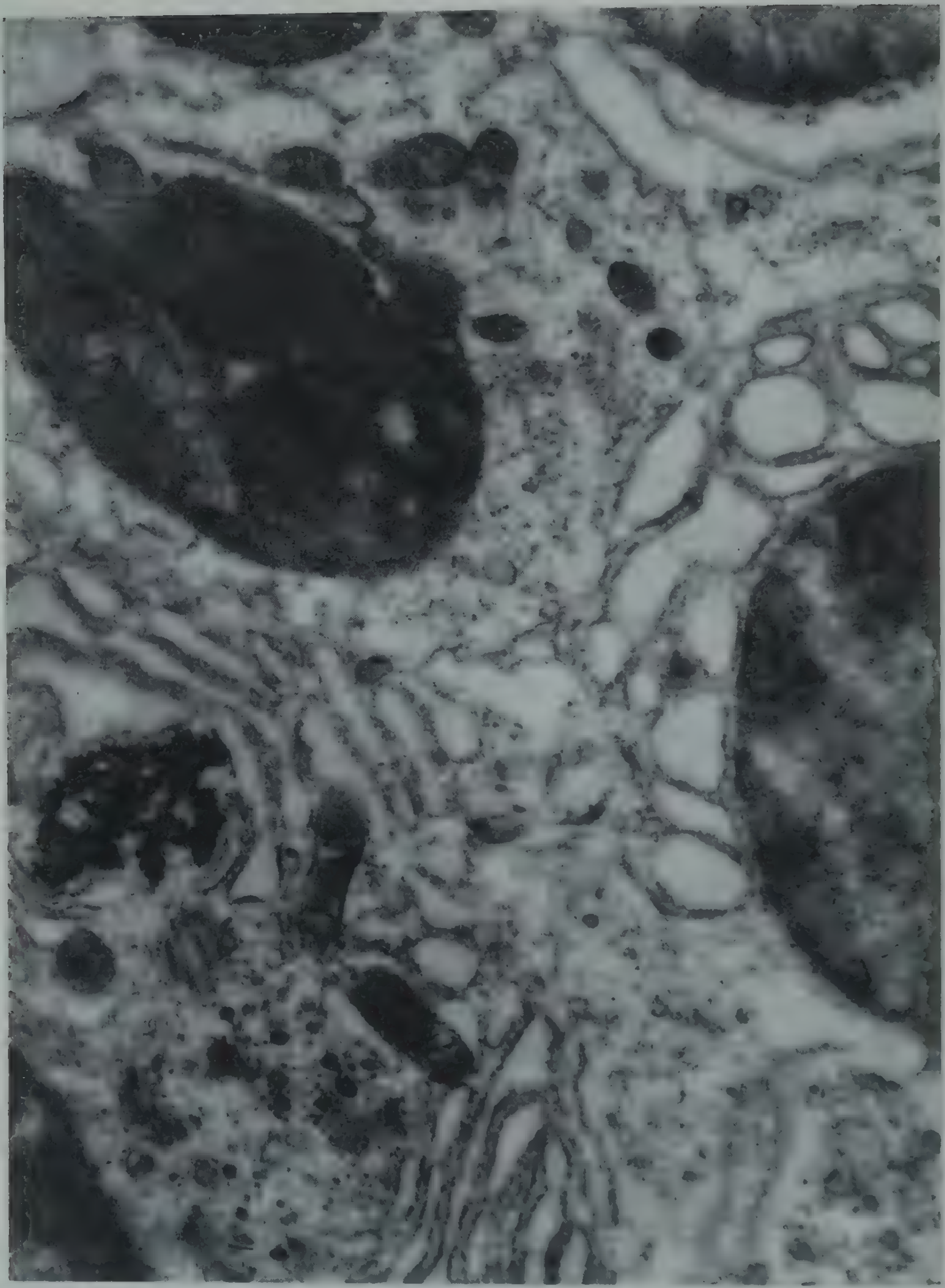


Fig. 13. Electron micrograph of thinly sectioned plasma cells from mouse spleen fixed with osmium tetroxide. The cytoplasms of these cells are filled with many membranes and canaliculi (endoplasmic reticulum) covered with RNA granules. Magnification: × 22,000.

proof that plasma cells are the only ones capable of synthesizing antibodies or any absolute conclusive proof that they are the principal cells concerned. Although antibody is not to be found within lymphocytes in any appreciable amount, a role of these cells in the production of antibody cannot be excluded. Indeed the participation of lymphocytes in the origin of antibody cannot be doubted, although as yet the mechanism of this participation is obscure.

As in the protein-synthesizing liver cells noted above, the plasma cells are in direct contact with the blood. It is not necessary for the proteins contributed by these cells to pass through an endothelial barrier in reaching the blood.

B. Animal Sera

1. Embryonic Development

In both fowls and mammals, the embryonic and fetal plasmas differ widely from those of the adult. Electrophoretic analyses show rapid changes during this developmental period (48). The concentration of nondialyzable, refractive material is at first very low. The plasma of 13-day White Leghorn embryos contained 0.66 mg. nitrogen per milliliter. After prolonged dialysis against water, freeze-drying, and then vacuum-drying until constant weight, 6% nitrogen and 2% ash were found. Both chick and pig embryonic plasma were found to be relatively rich in carbohydrates, which did not vary appreciably as the protein increased with development. Essentially a single component with a molecular weight of more than 200,000 was indicated by sedimentation and diffusion data on 11-day-old chick embryo serum. Pig fetal serum contained much smaller molecules (molecular weight approximately 60,000).

Electrophoretic data for developing chick and pig embryonic plasma are given in Tables IV and V, respectively. Components with mobilities similar to α- and β-globulins are predominant in the plasma of the early embryos, with the albumin steadily increasing during the developmental period. The component with the mobility of γ-globulin (No. 5) does not appear until rather late.

Using eggs from New Hampshire breed chickens and a borate buffer of pH 8.6, Heim and Schechtman (49) found components with mobilities faster than albumin occurring between 10 and 18 days of incubation. The fact that the nondialyzable components of embryonic plasma are very different from those found in the adult may mean that the organs and tissues which synthesize them are not sufficiently developed to produce the adult plasma products. The carbohydrate-rich constituents serve only as a temporary measure during the rapid developmental period. During the last stages of fetal development of the chick there is a substantial increase in the amount of the component with the mobility of γ-globulin. In mammals there is a correlation between the amount of fetal γ-globulin and the thickness of the placental wall. Those which have complex placental

TABLE IV

Electrophoretic Fractionation of Chick Embryo Plasma[a,b]

Exp. No.	Medium	Dilution	Mobility × 10^5 cm.^{2}/volt-sec. A$_1$	A$_2$	G	O	C	Composition (%) A$_1$	A$_2$	G	O	C
1	Egg white	10 ×	4.7	4.2	2.6	1.9	1.0	60	10	7	8	15
	Embryo Plasma (age in days)		Fraction number 1	2	3	4	5	1	2	3	4	5
2	8	2 ×	5.8		3.4			10		90		
3	9	3 ×	5.4		3.4			15		85		
4	10	3 ×	5.8		3.8	2.2		25		70	5	
5	11	2.2 ×	5.5		3.5	1.7		30		60	10	
6	13	3 ×	5.5		3.9	2.3		60		35	5	
7	17	None	5.5	4.7	2.8	2.4	1.6	55	10	8	13	14
8	+3[c]	2.4 ×	5.1	4.7	2.9	1.9	1.5	35	25	10	25	5
9	Adult	4 ×	5.3	4.4	3.5	2.5		40	25	5	20	10

[a] Buffer: 0.02 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4.

[b] From Moore *et al.* (48).

[c] +3 = 3-day-old chick.

walls like pigs or cattle are born with little γ-globulin and develop it rapidly after the ingestion of colostrum, whereas the newborn of mammals with thin placental barriers, like man and most rodents, have γ-globulins in concentrations equal to that of the mother.

TABLE V

Electrophoretic Fractionation of Pig Embryo Plasma[a,b]

Exp. No.	Embryo length mm	Dilution	Mobilities × 10^5 cm.^{2}/volt-sec. 1	2	3	4	5	6	Composition (%) 1	2	3	4	5	6
1	30	4 ×	6.2	5.0	3.9		1.9		10	50	15		25	
2	130	2 ×	5.3	4.5	3.5	2.5			35	35	20	10		
3	160	2 ×	5.1	4.3	3.8	2.7			45	40	10	5		
4	180	None	4.7	3.5		2.7	1.6		45	40		10	5	
5	250	None	4.5	3.3		2.4	1.7		55	35		7	3	
6	300	2 ×	4.6	3.3		2.5		0.9	50	30		5		15
7	Adult	4 ×	4.8	3.3		2.4		1.2	50	15		15		20

[a] Buffer: 0.02 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4.

[b] From Moore *et al.* (48).

2. *Normal and Experimental Animal Sera*

Many factors may affect the distribution of plasma components in electrophoretic patterns. It has been demonstrated that patterns are different in each of several species and that these differences are characteristic and reproducible when blood is taken from animals living in the same colonies and is studied under uniform conditions. However, such factors as strain, care, age, sex, fasting, and thirsting all affect the electrophoretic pattern.

Serum patterns of twelve species are illustrated in Fig. 14 (50). Most of the serum samples were taken from animals living on similar diets under similar conditions, and it is possible that sera from the same species living under different conditions might present a pattern different from the one here represented as typical. However, serum patterns of cotton rat and guinea pig obtained in 1957 have the same general characteristics as those obtained in 1945 (Fig. 15).

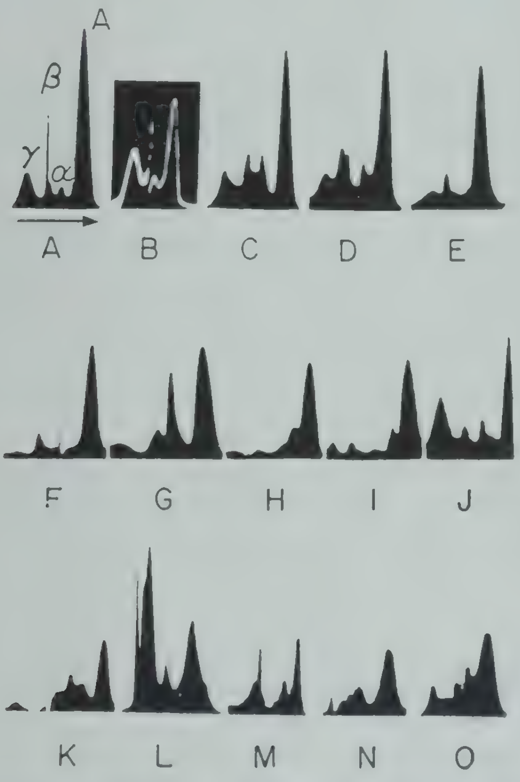


Fig. 14. Electrophoretic patterns of serum from various species. Phosphate-saline buffer, pH 7.4; ionic strength, 0.2. A, man; B, monkey; C, swine; D, dog; E, rat; F, 21-day-old rat; G, cotton rat; H, guinea pig; I, hamster; J, cat; K, 4-day-old kitten; L, chicken, hen; M, cock; N, female pigeon; O, carp (50).

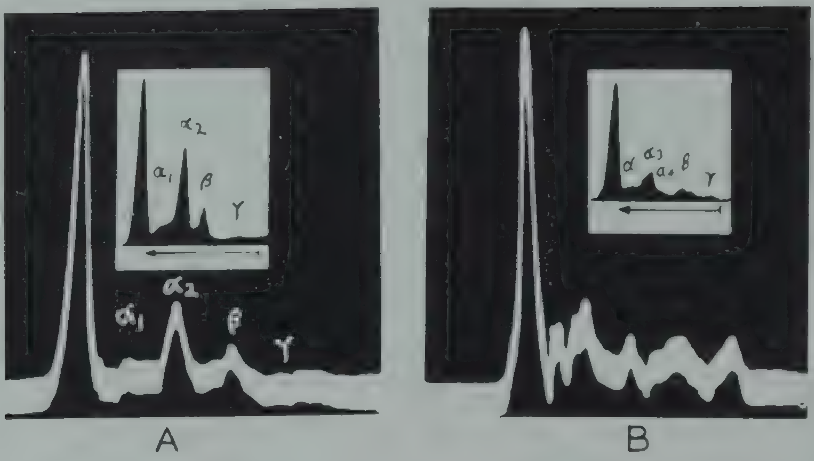


FIG. 15. Patterns of cotton rat, A; guinea pig, B. Serum analyzed in diethyl barbiturate buffer, pH, 8.6, ionic strength 0.1. Large patterns were obtained in 1957, whereas the inserts were made in 1945. They contain essentially the same characteristics. The initial boundaries for the large patterns were artificially sharpened. This procedure provides better resolution for the many components of these sera.

Halliday and Kekwick (25) studied the component levels of rat serum during the first 90 days of life. They found a component moving ahead of the albumins—possibly a second albumin—(buffer: 0.0044 M KH_2PO_4 + 0.0652 M Na_2HPO_4, pH 8.0) which varied in concentration from 8.6% of the total serum at 12 days of age to 4.2% at 90 days. During this period the total albumin rose from about 60% to about 70% of the total protein. These authors also presented evidence that maternal antibodies were absorbed by the gut of these young animals until the twentieth day after birth whereupon the γ-globulin level dropped and the β-globulin rose. Marked variations in so-called normal rat sera are recorded in the literature (51, 52); however, differences in technique (paper versus free) account for part of the discrepancies. It is often difficult to recognize a normal healthy animal, and those with low albumins and high globulins probably should not fall into this category, because it has been well established that the highest albumin to globulin ratios are associated with the highest state of health and that all diseases and abnormalities, if reflected in the serum at all, cause a decrease in the albumin to globulin ratio. It is possible that the low albumin and high globulin of such animals as monkeys and cats result from the fact that these animals are poorly adapted to laboratory cage life, and the similar characteristic of most horse serum may result from the general state of health of horses usually taken for antibody production or experimental purposes. The patterns of young rats and young cats in Fig. 14 indicate a higher albumin to globulin ratio than do the adults of these species, the γ-globulins being significantly lower in the young.

As animals grow older there is usually an increase in γ-globulin, and

Wehmeyer (53), in studying guinea pigs, associated this with the generally poorer biological quality of the older animals. Such changes with age have also been reported for sheep (54) and for rats and man (55).

Sex differences in serum of the domestic fowl become evident at about five months of age (56). These differences are primarily due to lipids or lipoproteins as influenced by the sex hormones. Serum patterns of hens and capons have been essentially reversed by injecting the hens with testosterone propionate and the capons with α-estradiol dipropionate, as is illustrated by the patterns of Fig. 16.

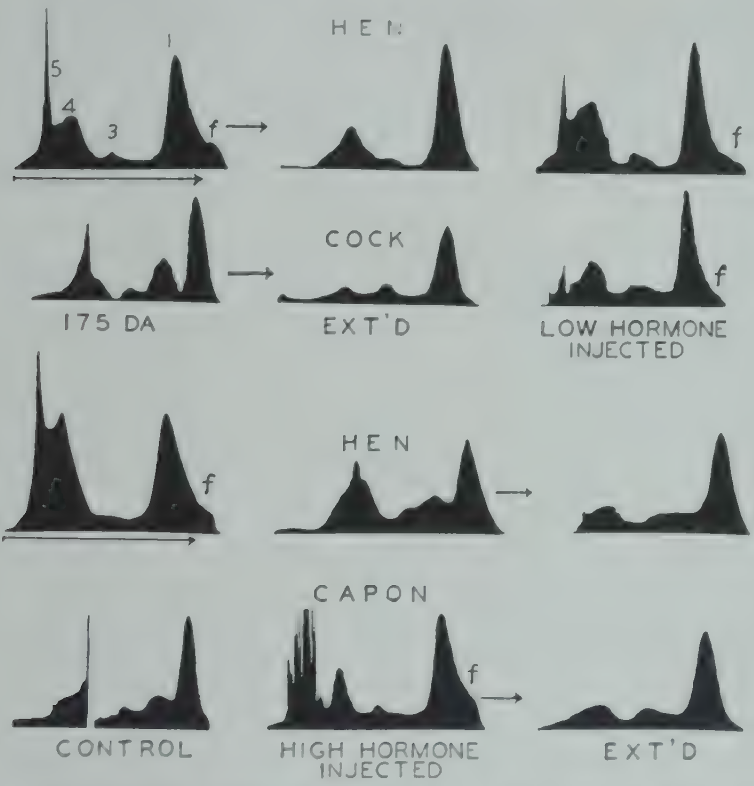


FIG. 16. Electrophoretic patterns of female and male chicken sera showing difference after maturity (175 days); same sera ether extracted (ext'd), changes produced by 0.2 mg. testosterone propionate and 0.1 mg. α-estradiol dipropionate daily for 14 days in the hen and cock, respectively (low hormone); control hen and capon at 260 days (control); after 1 mg. per day in both hen and capon for 18 days (high hormone); and same sera ether extracted (ext'd). A component ahead of albumin is indicated by f (56).

Dietary protein deficiencies in dogs cause a marked decrease in the albumin (57, 58). Dogs maintained on a protein-free diet for 30 days with plasmapheresis on the 10th, 11th, and 17th days gave serum patterns indicating 30–70% reduction in albumin without much change in the globulins.

except that there was an increase in the lipids associated with them. During the period all of the dogs lost weight and showed a decrease in plasma volume.

After acute depletion by plasmapheresis where one-half the total plasma proteins was removed within an interval of 30 minutes, the relative proportion of the electrophoretic components was disturbed for as long as 2 or 3 weeks after the total protein level had returned to normal (59). During the first 24 hours appreciable quantities of all components entered the blood stream. Alpha and beta globulins and fibrinogen returned most rapidly and were usually above normal within 2 to 3 days. Albumin and γ-globulins returned to their normal values much more slowly and required usually more than 40 days.

The effect of thirsting on the plasma of healthy and culled cows is given in Tables VI and VII (60). Within 2 hours after intake of water after 3 days of thirsting all the animals showed a marked fall in albumin concentration. In the healthy group of five cows the decrease in albumin concentration was accompanied by a rise in the globulin concentration. In another group of normal cattle followed over a prolonged period of time, Wehmeyer (61) found that the absolute concentrations of albumin and of globulin appeared to vary independently of each other and that sera with high total protein concentrations had high γ-globulin concentrations, and vice versa. No relation was found between the fibrinogen concentration and the globulin concentration. These data stress the great care required in assigning small changes in the plasma pattern to specific causes.

TABLE VI

CONCENTRATION OF PLASMA PROTEINS AND CELL VOLUME DETERMINED IN A HEALTHY COW DURING THIRST AND AFTER INTAKE OF WATER[a]

	Not fasting	Thirsting	½ Hour after intake of water	2 Hours after intake of water
Protein concentration (%)	7.70	8.48	7.82	7.98
Albumin concentration (%)	3.40	4.16	3.12	3.20
Globulin concentration (%)	4.30	4.32	4.70	4.78
Fibrinogen concentration (%)	0.59	0.69	0.63	0.63
Cell volume (%)	36	44	38	37

[a] From Wehmeyer (60).

Lethal doses of nitrogen and sulfur mustards in dogs caused the appearance of new spiked components and other increases in the α- and β-globulins

TABLE VII

Concentration of Plasma Proteins Determined in a Culled Cow during Thirst, Hunger and after Intake of Water[a]

	Not fasting	Thirsting	½ Hour after intake of water	2 Hours after intake of water	Not fasting	Fasting 2 days
Protein concentration (%)	8.35	9.58	9.00	8.79	8.60	8.68
Albumin concentration (%)	3.22	4.07	4.02	3.30	3.07	3.18
Globulin concentration (%)	5.13	5.51	4.98	4.49	5.53	5.50
Fibrinogen concentration (%)	0.64	0.66	0.61	0.74	0.75	0.86
Cell volume (%)	20.6	21.7	24.0	17.9	21.3	25.9

[a] From Wehmeyer (60).

regions of the serum patterns (62). There are indications that these represent the presence of several new protein fractions, at least one being rich in lipids and another poor in lipids. There is an accompanying fall in albumin concentration. A variety of severe injuries (scald, bone fracture, etc.) to dogs caused marked elevation and a spiked appearance of the α- and β-globulin pattern within 3 to 7 days after injury, as is illustrated in Fig. 17 (63). Such injuries may cause the appearance of physiologically active materials in the blood, such as leucotaxine, leucocytosis-promoting factor, necrosin, and pyrexin (64). There may also be an increase in serum lipids and lipoproteins.

Different strains of mice have been found to have characteristically different serum patterns (65). Two strains and their response to infection with *Salmonella typhimurium* 4 days after inoculation are shown in Fig. 18. Strain S had only one β-globulin, whereas Strain E consistently showed

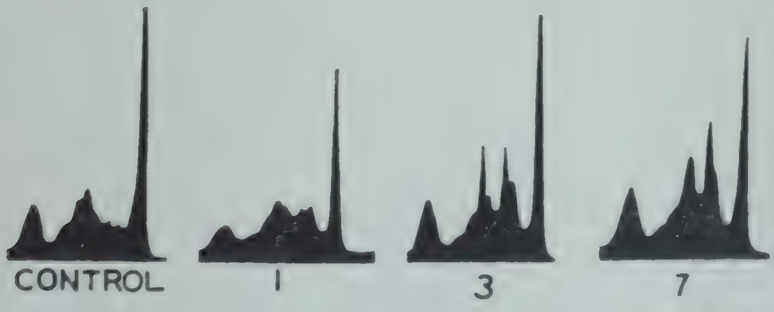


Fig. 17. Serum patterns taken before (control), and 1, 3, and 7 days after, a dog was scalded under anesthesia at 73° C. for 6 seconds. The head, shoulders, and forelegs were not exposed to the hot water (63).

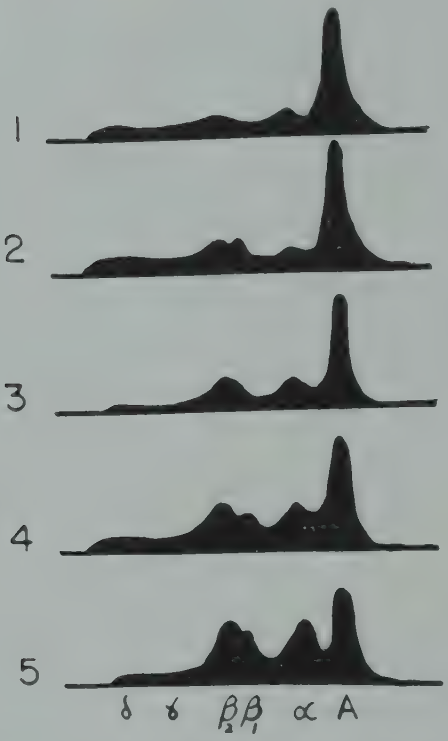


FIG. 18. Patterns showing strain difference in mouse sera analyzed in a phosphate-saline buffer at pH 7.8, ionic strength 0.2. 1, Strain S, normal; 2, Strain E, normal; 3, Strain S, infected with *Salmonella typhimurium;* 4, Strain E, infected with *Salmonella typhimurium;* 5, hybrid (S × E), infected with *Salmonella typhimurium* (65).

2 β-globulins. The α- and β-globulins were markedly elevated, particularly in the hybrid mice, after infection. The γ-globulins, however, remained quite low at this period of infection.

Although antibodies have often been found to be associated with γ-globulin it is quite evident that the total quantity of γ-globulin cannot be used as a criterion of immunity. Usually the total quantity of circulating antibody to any particular antigen is so small that its presence cannot be detected electrophoretically, and often repeated inoculations which result in hyperimmunity do not produce correlative changes in the electrophoretic pattern. If γ-globulin represents only antibodies, its elevation does not indicate protection from infectious diseases. For example, it has been found that the so-called normal rats of the Sherman strain at Columbia University have from 3 to 5 times as much γ-globulin as do the rats of the Long-Evans strain which are under different care. The Long-Evans rats are significantly hardier, faster growing, and have fewer infections than the other colony, which has the higher γ-globulin. It is still possible, however, that elevated γ-globulin represents a normal or an abnormal response to one or many specific stimuli (antigens). The occasional highly elevated globulin in hyperimmune sera seems to be a normal response to repeated stimulations by an antigen, but often only a small fraction of the increased γ-globulin can be demonstrated to be specific antibody to the antigen used. The changes in the pattern after immunization are not specific.

3. Distribution of Antibody in Serum Fractions

Although antibodies are usually associated with the γ-globulins, reports have claimed detection of certain antibodies in all the other electrophoretic fractions, even albumin (66). In hyperimmune horse sera the antibody activity against *Corynebacterium diphtheriae*, *Clostridium tetani*, sometimes *Streptococcus pneumoniae* and certain other bacteria has been found to be associated with a new "T" component which migrates between β- and γ-globulins (67–69). In syphilitic sera the fraction responsible for the positive serological reaction also migrated between β- and γ-globulin (70). Neutralizing antibodies to several strains of equine encephalomyelitis viruses were found in rabbit sera to be associated with either the β- or γ-globulin or to migrate between them (71). In chicken serum, where the usual electrophoretic components are difficult to recognize, encephalomyelitis antibodies were found to migrate with a mobility of 2.3×10^{-5} cm^2/volt^{-1}-sec.$^{-1}$ (phosphate buffer, pH 7.6; ionic strength, 0.2), which is that of one of the intermediate fractions (71).

Insulin-binding antibody in insulin-treated human patients was found by paper electrophoresis to migrate in the low-protein region between β- and γ-globulin (72).

Cann and Loveless (73) observed reagin activity in patients sensitive to cottonseed or ragweed pollen to be most closely correlated with β-globulin separated by electrophoresis convection. In examining the serum of an insulin-resistant diabetic patient who exhibited some insulin allergy as well, they (74) found that, whereas skin-sensitizing antibody was associated with the β-globulin, blocking antibody was contained in fractions of lower mobility (γ-globulin). Kuhns (75), however, found that skin-sensitizing antitoxin migrated as γ_1-globulin, whereas precipitating antitoxin migrated as γ_2-globulin. Menzel and co-workers (76 and more recent unpublished data) have shown that the skin-sensitizing antibody to ragweed pollen appears in several of the globulin fractions and that the distribution may vary from one serum to another. The nature of this reagin is as yet poorly defined. Accordingly, it may be concluded that antibodies are not always strictly confined to γ-globulin. They may be associated also with other globulins; they are most frequently found in γ_1-fraction or between β- and γ-globulin; and different types of antibodies may have different physicochemical properties.

C. Normal Human Plasma

The literature contains reports of numerous measurements on normal human serum. In the buffer system, 0.1 N sodium veronal-0.02 N diethylbarbituric acid, pH 8.6, more than a thousand measurements of normal human serum or plasma have been reported. The proportions of the various components of serum determined by the moving boundary method lie between the following values: albumin, 53 and 63%; α_1-globulin, 4.0 and

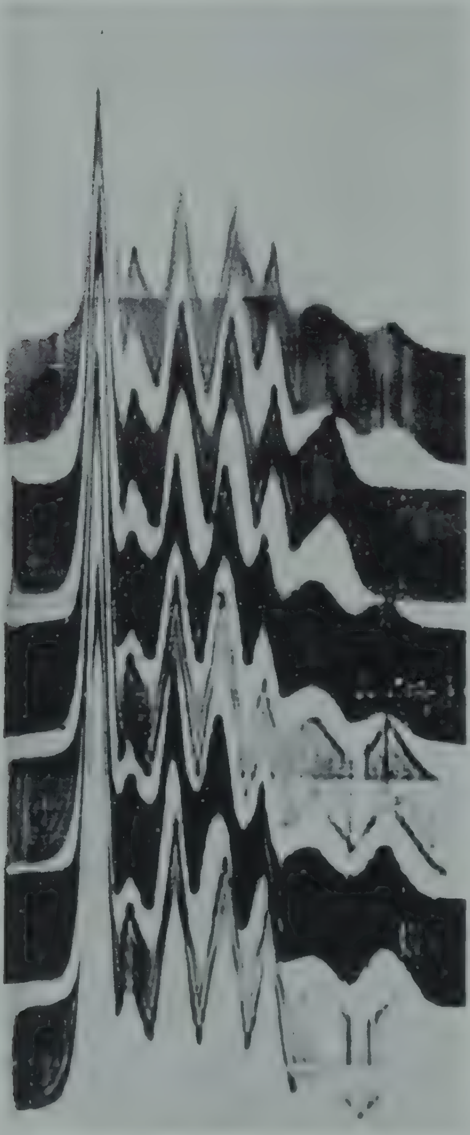


FIG. 19. Serum patterns from a patient with breast cancer taken at different times Dates from top to bottom: May 23, 26, 28, and 30; June 7 and 11. Veronal-citrate buffer, pH 8.6, ionic strength 0.1 (77).

8.0%; α_2-globulin, 8.2 and 10.4%; β-globulin, 10.8 and 14.7%; and γ-globulin, 8.5 and 14.7%. In round numbers the most probable values are about as follows: albumin, 58%; α_1-globulin, 5%; α_2-globulin, 9%; β-globulin, 14%; γ-globulin, 14%. When *plasma* is analyzed the amount of fibrinogen is usually a little larger than α_1-globulin, and because the total pattern is larger the above percentages are slightly smaller. It has already been noted that species and sometimes strains or colonies of animals have different and characteristic serum patterns. It has also been shown that there are characteristic individual electrophoretic patterns in humans, as is illustrated in Figs. 19 and 20. Bernfeld and associates (77) state that it was difficult to find two persons, either healthy or otherwise, whose patterns had the same characteristic features. The shape, symmetry, width, and spacing of each peak are of great importance in characterizing a given pattern. These features remain constant for the same individual over long periods of time, although the protein concentration and even the albumin to globulin ratio may change. Dole (78) has studied the plasma of a normal individual over a period of four months and has found that the components vary appreciably, as is indicated by the chart of Fig. 21.

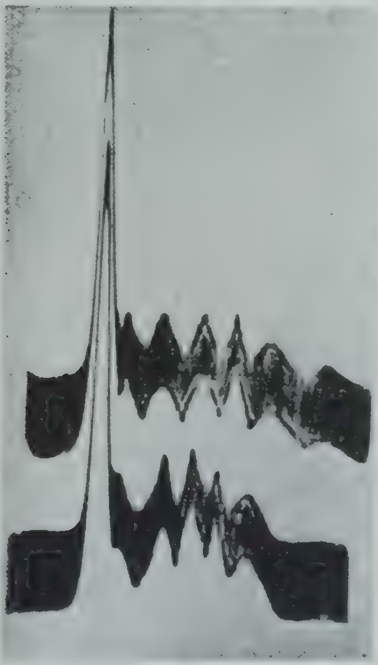


FIG. 20. Serum patterns from two different individuals. Although the quantitative measurements of the components are similar, the profiles are characteristically different (77).

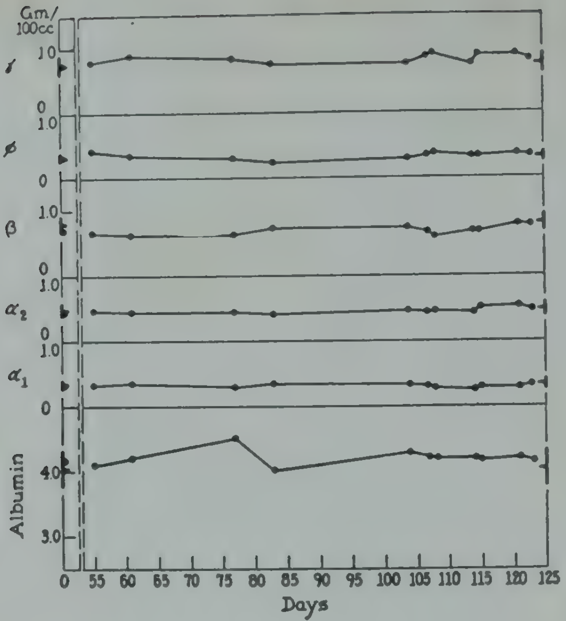


FIG. 21. Chart indicating the level of the plasma components of one normal individual over a period of four months. Mean values and component variations are indicated on the ordinates (78).

1. *Fetal Development of Human Serum*

As noted in a foregoing section there are marked species differences in the fetal development of the serum proteins. In man the barrier separating fetal blood from maternal blood consists, at term, of only a single cell layer, which is the thinnest barrier of any mammal except of certain rodents, e.g. the guinea pig. To answer the question of whether any of the fetal proteins come from the mother already synthesized or whether they are mostly synthesized by the fetus itself, let us consider the following data. The concentration of the various electrophoretic fractions of maternal and fetal sera from the third month to term are compared graphically in Fig. 22 (79). Although the total amount of refractive material in the maternal serum is greater than that in the fetal, both the albumin and γ-globulin in the fetal serum rise to a greater level at term (80). The albumin and γ-globulin increase rapidly during fetal development, whereas the α- and β-globulin remain at a constant low level.

In the maternal serum the α- and β-globulins rise appreciably and the albumin decreases during pregnancy. Therefore, if any serum proteins cross from maternal to fetal blood, the placenta must play an active role in selecting the components transferred because there is no parallelism between the variations that take place in the two sera. However, many investigators have demonstrated the presence of antibodies in newborn

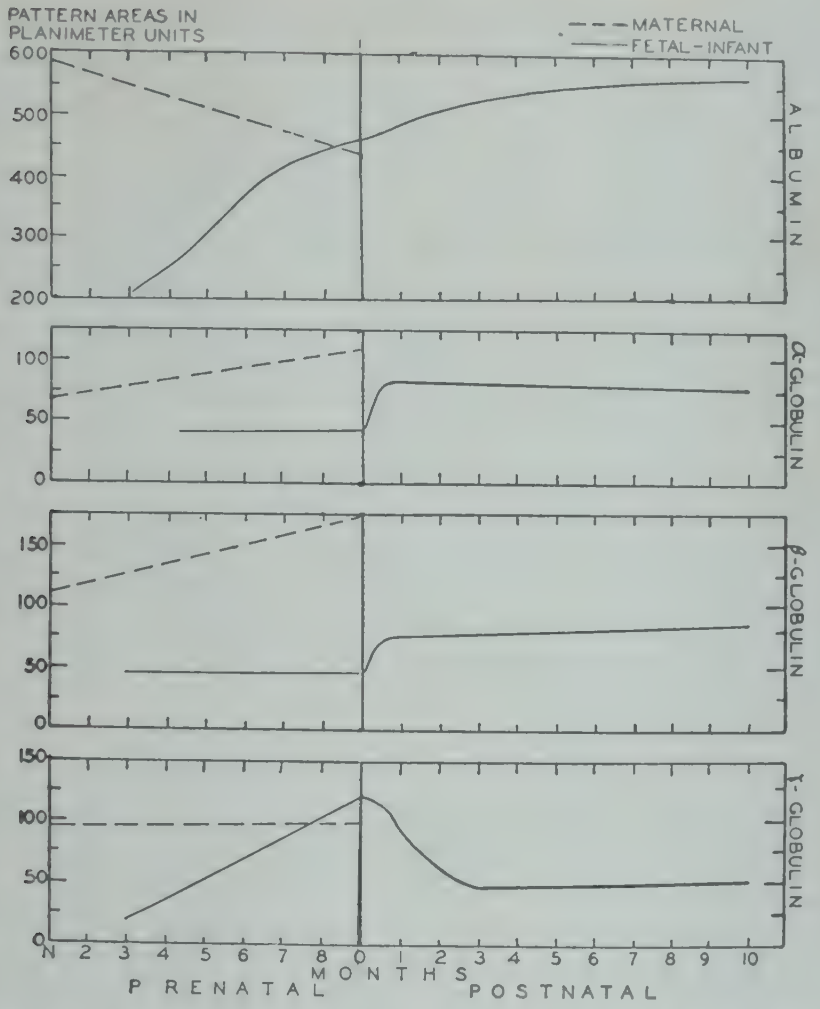


FIG. 22. Chart indicating the development of serum proteins in the human fetus and during the first ten months of postnatal life. Relative concentrations of maternal serum components during pregnancy are also shown (79).

infant serum, and no doubt these antibodies are a part of the γ-globulin. Moreover, Pommerenke has shown that the venous blood of the umbilical cord contained significantly more globulin than the arterial blood (81). Other evidence for the placental transfer or placental synthesis of γ-globulin is offered by the postpartum data of Fig. 22. The decrease in γ-globulin during the first few months of postnatal life corresponds to the antibody disappearance rate one would expect from the work of Wiener (82), who found that in the newborn infant the half-life of an antibody molecule was about 30 days. Assuming such a decay rate with some postnatal replenishment, the γ-globulin curve of Fig. 22 might be expected. Other methods of measuring antibodies and γ-globulin of infant serum also indicate decreases according to the postpartum γ-globulin curve.

The above circumstantial evidence for the placental transfer of maternal γ-globulin has now been substantiated by a more direct approach employing metabolizing cultures from human fetuses and placentas (83). Liver slices from fetuses of 3 to 4 months and at term were incubated in Krebs-Ringer solutions containing glycine-*2*-C^{14}. After 18 hours of incubation the proteins were separated electrophoretically and immunologically, and the incorporation of radioactivity was determined. All of the plasma proteins were present except γ-globulin. On the other hand, 3- to 4-month-old placentas produced only proteins immunologically unrelated to the plasma proteins although components containing radioactive glycine with the mobilities of α- and β-globulins were present. However, at term these placental proteins were related immunologically to plasma globulins. Similar studies were conducted using human fetal heart. It was found *not* to synthesize plasma proteins.

There now seems to be adequate proof that the fetal liver synthesizes the fetal albumin but that neither the fetus nor the placenta synthesizes γ-globulin (at term the placenta probably synthesizes some α- and β-globulins). The γ-globulin must, therefore, be selectively transferred from the maternal to the fetal blood.

D. Pathological Human Plasma

This section might appropriately be started with a question. Considering the present knowledge of sources and sinks of plasma proteins, the pathology of what organs or cells is likely to be reflected in the composition of plasma? It would seem that alterations in the plasma should result from primary alterations in protein-synthesizing organs (liver, spleen, lymph nodes, bone marrow) and/or alteration in its disposal. Of course, there can be secondary effects upon these and other organs especially in chronic diseases which cause, e.g. an alteration of the liver cells which synthesize albumin; or a malignant disease such as multiple myeloma may not only produce an abnormal protein but also cause an alteration in the kidney so that albumin is lost into the urine. It is also possible that cells which do not normally produce any of the plasma components could be altered in disease so that they do produce either existing or new components. For the most part it is these indirect effects which make the plasma picture difficult to interpret.

There are a few diseases, however, where a direct correlation between pathology and plasma pattern is quite good.

1. Kidney Diseases

The serum pattern seen in lipid nephrosis, Fig. 23, is probably the most characteristic and the most typical of any pathological condition studied

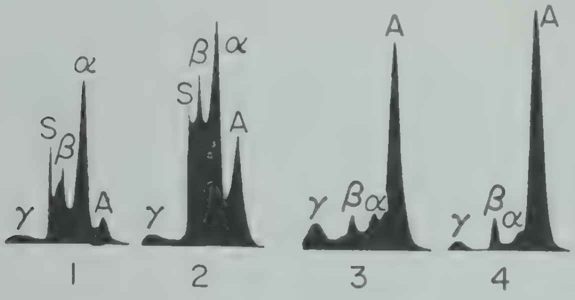
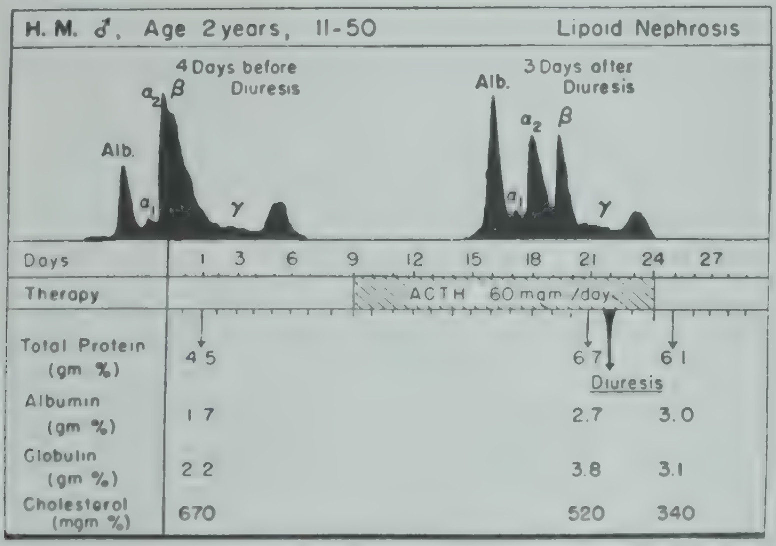


FIG. 23. Patterns of nephrotic and corresponding urines. 1, serum from patient S. G.; 2, serum from patient G. B.; 3, urine from patient S. G.; 4, urine from patient G. B. Lithium barbiturate-lithium chloride buffer, pH 7.8, ionic strength 0.05 (84).

(84). The writer is unaware of any case of this disease which does not produce this general type of pattern. The large elevations in α- and β-globulin parallel the elevation of lipids and cholesterol. When the serum is clear usually only cholesterol is increased, whereas neutral fats cause the opalescence of lipemic sera (85). The urine of these patients contains a large amount of albumin and the electrophoretic pattern often resembles that of normal serum, as is seen in Fig. 23. In lipid nephrosis it is possible to cause diuresis in many patients with a variety of drugs such as ACTH, cortisone, urea, cation exchange resins, and nitrogen mustard. Electrophoretic patterns of the serum before and after treatment with ACTH are shown in Fig. 24 (86). After diuresis there is a marked increase in albumin accompanied by a decrease in the greatly elevated, often unresolved, α- and β-globulins.



Total Protein (gm %)	4.5	6.7	6.1
Albumin (gm %)	1.7	2.7	3.0
Globulin (gm %)	2.2	3.8	3.1
Cholesterol (mgm %)	670	520	340

FIG. 24. Serum patterns of a patient with lipid nephrosis before and after diuresis caused by ACTH (86).

2. *Liver Diseases*

Since the liver is normally the site of synthesis of albumin and a part of the globulin, any alteration in liver function is likely to be readily reflected in the serum. Diseases which cause metabolic stress may cause liver damage and therefore changes in the serum pattern. It turns out that practically all diseases and disorders have a tendency to lower the concentration of albumin and many of them elevate the concentration of one or more of the globulins.

There has been established a good correlation between degree of hypoalbuminemia and severity of liver necrosis (87). In chronic liver diseases the albumin falls to a very low level coincident with the replacement of parenchymal cells by fibrous tissue. Chronicity of liver disease has a more marked influence on the albumin depression than severity (88). Wajchenberg *et al.* (87) have found that the sera of patients with diffuse, massive necrosis were characterized by the appearance of an α_3-globulin migrating between α_1- and α_2-globulins which did not appear in liver diseases such as benign viral hepatitis, cirrhosis, and hepatoma. There is little correlation of α_1- and α_2-globulins with severity or type of liver disease although α_2 fluctuates widely. The β-globulins were found to be of great diagnostic value, because when they are very low the prognosis is unfavorable. The β-globulins and the lipemia decrease when massive necrosis has destroyed cells active in protein synthesis.

In cases of cirrhosis Franklin *et al.* (89) have found that the apparent elevation of fibrinogen is really due to the inclusion of a γ_1-globulin which has about the same mobility. In almost all liver diseases the γ-globulin is elevated. It is also broadened, indicating a heterogeneous group of globulins. In cirrhosis it decreases as the patient responds to dietary correction (90). The increase in γ-globulin is thought to be caused in part by the mesenchymal reaction of the Kupfer cells and histocytes of the portal spaces to the stimulus furnished by the products of decomposition of the parenchymal cells (87). In infectious hepatitis a part of the γ-elevation may also be due to viral antibody formation.

In viral hepatitis the positive cephalin-cholesterol flocculation reaction of Hanger is primarily due to alteration, probably both quantitative and qualitative, of the serum albumin (91). Isolated γ-globulin from both normal serum and serum from cases of hepatitis cause the cephalin-cholesterol emulsion to flocculate. Electrophoretically separated albumin from normal serum, however, tends to inhibit the flocculation of γ-globulin, whereas the albumin fractions from sera of hepatitis cases show relatively less inhibition

3. Multiple Myeloma

Although multiple myeloma does not produce a uniformly characteristic serum pattern it often causes marked deviates from the normal. Most cases fall into one of the following three classes: (a) sera with hyperglobulinemia due to elevated γ-globulin; (b) sera with or without hyperglobulinemia, giving a variety of anomalous patterns due mainly to the presence of Bence-Jones proteins; (c) normal pattern.

The albumin may be normal or it may be low. Sometimes it is very low. Often the Howe method of fractionation shows a normal or even elevated albumin because of the presence of Bence-Jones or other abnormal proteins which do not precipitate with the globulins. Electrophoresis seems never to confuse these abnormal proteins with albumin but always places them in the region of the globulins, sometimes with α-, sometimes with β-, and sometimes with γ-globulin, and sometimes near γ-globulin as an M-component. Urinary Bence-Jones protein also has a variety of mobilities (92, 93). The sedimentation constant of urinary Bence-Jones protein varies from one individual to another. Values of 2.8S, 3.4S, and 3.7S have been reported. This protein seems to be composed of smaller molecules than any of normal serum proteins (92). Some cases with sera showing abnormal β- or M-components have been shown by immunochemical and ultracentrifugal analyses to have Bence-Jones protein as the chief abnormal component of the serum (92). When the hyperglobulinemia is due to an elevated γ-globulin, the abnormal globulin has not been demonstrated to be Bence-Jones protein, but has a sedimentation constant of 7S and resembles normal γ-globulin. The turnover rate of urinary Bence-Jones protein has been found to be extremely rapid with a half-life of 1.5 days whereas the half-life of the greatly elevated (10 gm. per cent) serum γ-globulin in the same patient was 21 days (94). The electrophoretic mobilities, the molecular weights, and the carbohydrate content of the two proteins were all different. The relationships of these two, frequently characteristic, myelomatous proteins is not understood; however, it is not likely that the γ-globulin is a precursor of the Bence-Jones protein, because the isotopic nitrogen (N^{15}-l-aspartic acid) concentration maximum in the urinary protein preceded and exceeded the maximum in the serum γ-globulin.

In a series of 91 patients studied by Reiner and Stern (95), 22% showed insignificant abnormalities in the serum pattern, 6.6% revealed an abnormal component in the α-globulin region, 15.4% in the β-globulin region, and 55% had abnormal components in the γ-region. Renal impairment is frequent in multiple myeloma. It may or may not alter the serum pattern. The albumin or other serum proteins besides Bence-Jones protein, which occurs in the urine of about half the patients, apparently results from kidney damage. However, normal serum patterns are found in many patients who excrete large amounts of Bence-Jones protein.

4. Hypogammaglobulinemia

A great deal has already been said about the origin and significance of γ-globulin. It is now evident that the absence or near absence of this component is associated with a definite pathological condition. Three forms of this metabolic disturbance have been described (96): (a) transient form, found in infants; (b) congenital form inherited as a sex-linked recessive characteristic occurring only in males; and (c) acquired form, occurring in both sexes, with the onset of an infection in adolescence or adulthood. The latter two forms appear to be due to the absence of plasma cells, which have been established as the chief source of antibodies (97, 98; see also Section I, A, 3, a). Unlike the transient form, the failure of γ-globulin synthesis in the congenital and acquired groups, once established, seems to be permanent. Patients whose γ-globulin is very low may have electrophoretic patterns which indicate a complete absence, as in Fig. 25; however, traces can always be demonstrated serologically. Usually there are also deficiencies in other proteins which normally move as β-globulin. In children, hypogammaglobulinemia is associated with lack of resistance to infection and recurring sepsis (99). In some adults, however, the patient may stay un-

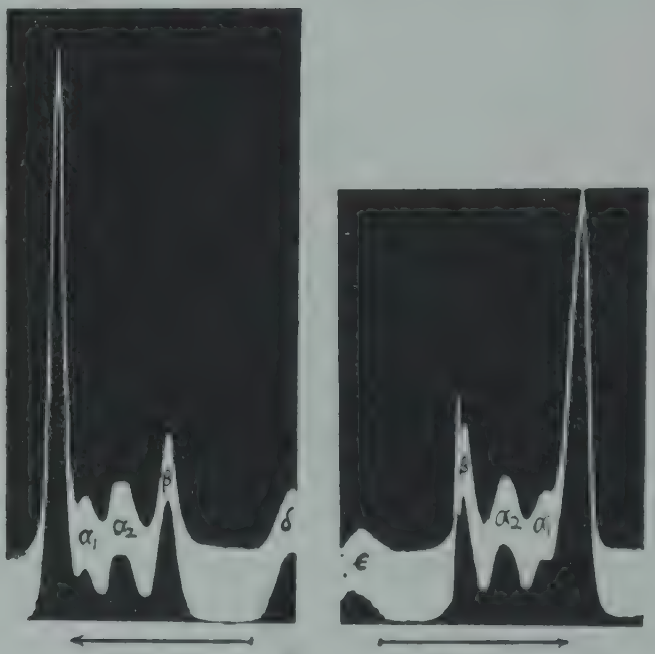


FIG. 25. Agammaglobulinemia serum from a 7-year-old boy. The pattern is normal except for the absence of γ-globulin. Traces, however, could be detected serologically.

usually free of infections in spite of a very low γ-globulin level (100). It was stated above (Section I, B, 3) that many if not most of the antibodies whose mobilities have been determined migrated between β- and γ-globulin where the concentration of protein is very low. It is therefore not surprising that there is not a good direct correlation between immunity and level of γ-globulin.

5. Hypergammaglobulinemia in Children

Children with recurrent or chronic infections have sometimes been found to have abnormally high γ-globulin with serum concentrations ranging from 1.5 to 6 gm. per cent (101). These children usually have enlarged spleens and livers and an excessive number of plasma cells in the bone marrow. This disease constitutes an immunologic paradox. It becomes evident very early in life and probably represents a genetic defect.

6. Diseases in General

The literature contains a myriad of reports on electrophoretic analyses of serum from man and animals with almost every known disease and disorder. Most of the diseases produce abnormalities in the serum pattern, but they are nonspecific. There is almost always a decrease in albumin and an increase in one or more of the globulins. It is not enough simply to measure patterns. It is also necessary to understand what mechanisms are involved in producing the abnormalities, whether the elevated globulin is due to protein, lipid, or carbohydrate, or a combination of them, whether it is a new kind of substance or simply more of the one that is already there in the normal state, and what organ or tissue produces the new material. This has been done in a great many instances and has proved to be helpful in diagnosing and understanding disease processes, as is indicated in the selected examples described above. Most of the work in this area, however, remains to be done. The following questions may be asked, as examples of needed information. Why do acute infections or febrile diseases cause a rise in the α-globulin? Could the cytoplasmic components of lymphocytes which are then disintegrating at a tremendous rate be partly responsible? Or is there simply an increase in the transport of bound lipids and carbohydrates? How are hormones linked with serum glycoprotein levels? Present knowledge indicates that a variety of hormones may markedly influence both bound carbohydrate and lipid levels as well as protein levels (102). In disseminated lupus erythematosis what is responsible for the greatly elevated γ-globulin which often occurs during the active phase of this disease (103)? There are indications that lupus erythematosis may result from an allergic hypersensitivity (104), but the cause of the marked hypoalbuminemia and elevated α_1-globulin often associated with this disease, as well as the nature of the elevated γ-globulin, are not understood.

II. Body Fluids and Related Substances

A. *Cerebrospinal Fluid*

By concentrating 70–80 ml. of spinal fluid, by pressure dialysis, to about 2 ml. the protein concentration becomes great enough to lend itself to free electrophoretic analysis. Spinal fluids and sera from the same patients are compared in Fig. 26 (105). There are similarities between the two substances which lead to the conclusion that at least part of the proteins of the spinal fluid are derived from the blood. Alterations in the serum components are reflected in the spinal fluid, but the changes are usually not as marked. In a case of neurosyphilis, however, where the serum pattern was essentially normal the spinal fluid showed a γ-globulin comprising 38% of the total protein (Fig. 26, J). Cerebrospinal fluids frequently show a significant amount of a component with a mobility greater than albumin (105–108). In some cases two small components with mobilities greater than albumin have been observed and in others a component migrating between β- and γ-globulin have been noted (106, 109). A true parallelism between cerebrospinal fluid and blood is lacking, but it must be remembered that in these experiments the spinal fluids were concentrated 20–40 times. Components of the spinal fluid are not present in the

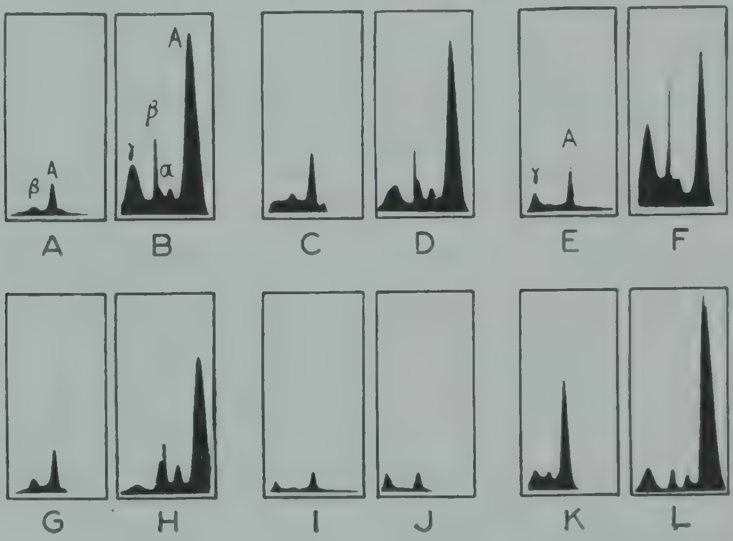


Fig. 26. Patterns of cerebrospinal fluids and sera. The spinal fluids were concentrated by pressure dialysis. A, spinal fluid; B, serum—idiopathic grandmal (normal); C, spinal fluid; D, serum—anxiety state (normal); E, spinal fluid; F, serum—lymphopathia venereum; G, spinal fluid; H, serum—multiple myeloma; I, spinal fluid—multiple sclerosis; J, spinal fluid—neurosyphilis; K, spinal fluid—diabetic neuritis; L, cyst fluid—left frontal cystic astrocytoma (105).

same proportion as in serum, but it has not yet been demonstrated that they are qualitatively different. However, some of the spinal fluid components may be formed within the tissues of the central nervous system. Antibodies and other globulins may be synthesized by reticulo-endothelial cells of this system.

B. Lymph

The subject of lymph has recently been thoroughly reviewed by Yoffey and Courtice (110). Suffice it to say here that lymph is very similar to plasma, containing the same proteins that plasma does, but at a lower level (111). The concentration of proteins varies in the lymph from different parts of the body, being highest in liver lymph and lowest in lymph from the skin. The albumin to globulin ratio in lymph is consistently slightly higher than in plasma. A great deal of evidence has accumulated indicating that essentially all of the lymph is derived from the blood. The plasma, consisting of salts, sugar, and the plasma proteins, filters or is in some way transported through the capillary walls into the tissue spaces. Some of the water and salt may be reabsorbed by the blood vessels, but the proteins are returned to the blood by way of the lymphatics.

C. Synovial Fluid

Synovial fluid is a protein-containing dialyzate of blood plasma to which mucin, secreted by the synovial cells, is added. The blood plasma apparently diffuses through the synovial tissue spaces into the joint cavity (112). The proteins have been studied in considerable detail by Schmid and MacNair (113) and have been found to be of the same nature as those of plasma, as judged by ultracentrifugal analyses, separation by solubility, immunochemical methods, and ion-binding measurements as well as by electrophoresis. These extensive comparisons of plasma and synovial fluid proteins were made only on cases of rheumatoid arthritis, but it is likely that the comparison extends to normal subjects. However, the presence of special protein components only in the synovial fluid was not excluded. Patterns of a synovial fluid and the corresponding plasma are shown in Fig. 27 (114). The total protein concentrations of human fluids are in the neighborhood of one-half that of plasma, that of bovine fluid being much lower. There are often concentration differences in the various components and both Perlmann (114) and Platt (115) and their associates report a rapidly moving component often found in normal fluid. This component is possibly hyaluronic acid or a hyaluronic acid-protein complex. The relative concentration of albumin is sometimes higher, sometimes equal, and sometimes lower in the fluid than in the plasma. The concentrations of α_2 and γ globulin are also often at variance with those of plasma. The α_1-lipoprotein, measured as cholesterol, appears higher in the fluid than in the plasma. A correlation between electrophoretic patterns of joint fluid and severity

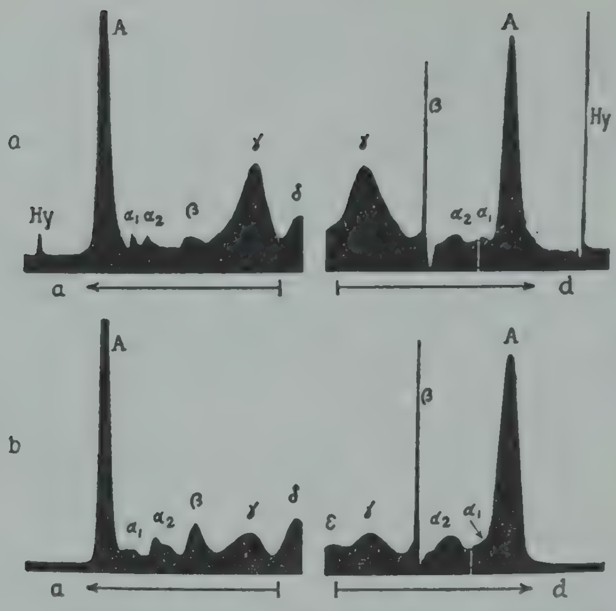


Fig. 27. Pattern of synovial fluid compared with serum from the same patient (114).

of disease is not good, although the more severe cases of joint injury tend to show a lower fluid albumin and higher γ-globulin than the serum.

D. Aqueous Humor

The low concentration of protein in the aqueous humor makes it necessary to concentrate the fluid before electrophoretic analyses. Pooled humors and individual sera of three species are compared in Fig. 28 (116). The patterns of rabbit and cattle aqueous humors reveal fast-moving components with mobilities as given in Table VIII. The fast component may be

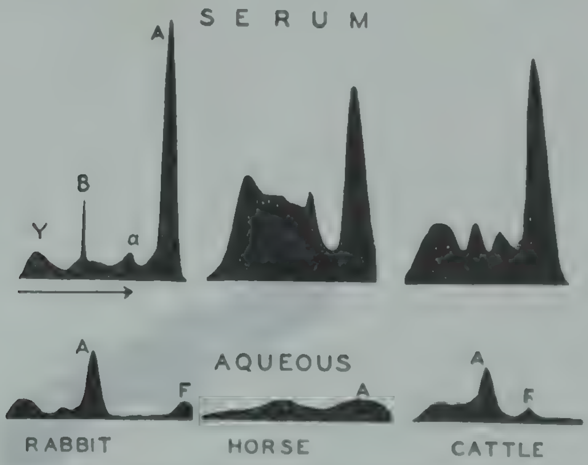


Fig. 28. Comparison of serum and aqueous humor from three species. The aqueous humors were concentrated by pressure dialysis. Phosphate-saline buffer, pH 8.4, ionic strength 0.2 (116).

TABLE VIII

ELECTROPHORETIC FRACTIONATION OF SERA AND AQUEOUS HUMORS OF RABBIT, HORSE, AND CATTLE[a]

			Percentage composition							Mobilities					
Species	Material	Treatment	Fast Component	Albumin	α-Globulin	β-Globulin	Fibrinogen	γ-Globulin	Albumin: globulin ratio	Fast Component	Albumin	α-Globulin	β-Globulin	Fibrinogen	γ-Globulin
Rabbit	Serum	Normal		56.1	12.7	17.2		14.0	1.27		5.25	3.36	2.79		1.08
	Aqueous		16.4	49.1	7.3			27.2	1.42[b]	12.2	5.96	3.82			1.18
Horse	Serum	Normal		36.7	12.3	21.6		29.4	0.58		5.30	3.44	2.50		0.96
	Aqueous			42.5		42.5		15.0	0.74		5.06		2.50		0.72
Cattle	Serum	Normal		53.2	9.5	11.7		25.6	1.14		5.20	3.63	2.63		1.25
	Aqueous		9.3	48.2	13.0			29.5	1.13[b]	9.6	5.50	3.28			1.18
Rabbit	Aqueous	After iontophoresis with histamine and physostigmine		61.5	14.1			24.4	2.34[c]		5.2	3.3			1.00
Rabbit	Aqueous	After injection of staphylococcus toxin		63.8	3.8	12.4	8.5	11.5	1.13		4.50	3.40	2.63	1.90	1.25

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[a] From Von Sallmann and Moore (116).

[b] Fast component omitted.

[c] Fibrinogen omitted.

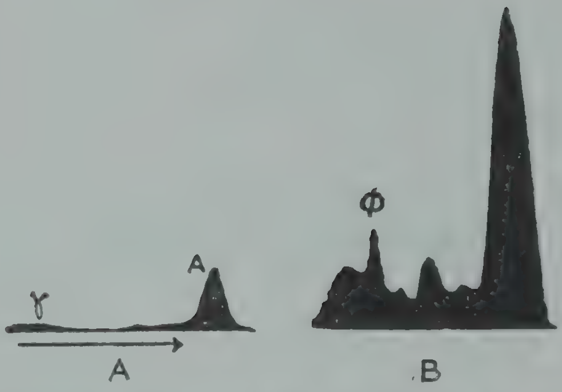


FIG. 29. Rabbit aqueous humor after eyes had (a) been subjected to iontophoretic treatment with solution of physostigmine and histamine, or (b) staphylococcus toxin had been injected. The aqueous humor for (a) was concentrated 2.9 times; that for (b) was not concentrated. Phosphate-saline buffer, pH 7.4, ionic strength 0.2 (116).

hyaluronic acid or a hyaluronic acid-protein complex. When rabbits' eyes were treated with vasodilators the albumin to globulin ratio was increased from 1.4 to 2.4 by the selective influx of albumin. Peaks for β-globulin and fibrinogen were absent from vasodilator-treated eyes (Fig. 29, A), whereas they were present in the patterns of the aqueous humor proteins of rabbits with endophthalmitis induced with staphylococcus toxin (Fig. 29B). In the acute stage of inflammation all components of the plasma proteins were drawn into the aqueous humor, whereas in the transient capillary dilation the vessel wall was rendered permeable to only the relatively smaller albumin molecules. The shift in the albumin : globulin ratio in the aqueous humor of moderately irritated eyes would seem to explain some of the

TABLE IX

RELATION OF PROTEIN CONCENTRATION IN MILK TO RATE OF GROWTH IN THE YOUNG OF VARIOUS SPECIES[a]

Species	Days required to double birth weight	Protein (gm. per 100 ml.)
Man	180.0	1.6
Horse	60.0	2.0
Cow	47.0	3.5
Goat	22.0	3.7
Sheep	15.0	4.9
Pig	14.0	5.2
Cat	9.5	7.0
Dog	9.0	7.4
Rabbit	6.0	10.4

[a] From Hawk *et al.* (118).

discrepancies between the colloidometrically measured intensity of the aqueous flare and the respective protein content of the aqueous humor of human eyes, because Tyndall scattering per unit nitrogen of albumin in solution is less than that for the other serum proteins (117).

E. Milk

The total amount of protein in milk varies greatly from one species to another. The concentration has been correlated with the growth rate of the young, as indicated in Table IX (118).

The proteins of cow's milk have been extensively studied. The eight principal components are characterized in Table X (119). Electrophoretic analyses show significant breed or strain differences, as well as individual variations (120).

Defatted milk can be readily clarified for electrophoretic studies by the addition of a small amount of sodium Versenate. The pattern of Versene-treated mouse (R III strain) milk, illustrated in Fig. 30 shows only four distinct components.

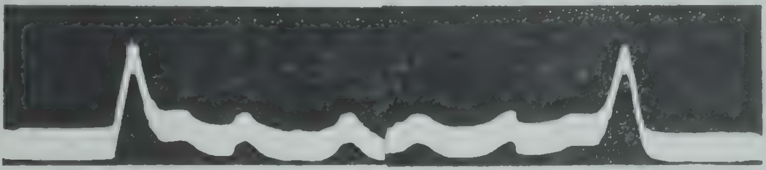


FIG. 30. Ascending and descending pattern of sodium Versenate-clarified milk from R III strain mice. Some components were present before treatment with Versene although the solution was too opaque to obtain a good pattern. Dilution 1:4 diethylbarbiturate buffer, pH 8.6, ionic strength 0.1.

Five electrophoretic fractions were found in the whey of human milk (120a). Electrophoretic, ultracentrifugal, and immunochemical studies indicated that serum albumin was present in the whey, but the presence of γ-globulin was not clearly demonstrated.

F. Seminal Plasma

The nature of human seminal plasma has been studied rather extensively. Cell-free plasma contains four and sometimes five distinct electrophoretic components when examined in a phosphate-saline buffer pH 7.85, ionic strength 0.10 (121). The quantity of these components varies widely from one individual to another. Under the conditions studied the average mobilities of the five components were 0.9, 2.9, 4.6, 5.7, and 6.3×10^{-5} cm^2/volt-sec., respectively. The slowest component (0.9) was non-heat-coagulable, passed through dialysis membranes (average pore diameter 25 Å) and was probably proteose. The next two components

TABLE X

INDIVIDUAL PROTEINS ISOLATED FROM COW'S MILK AND SOME OF THEIR PROPERTIES[a]

Protein	Classical fraction from which isolated	Approximate per cent of milk proteins[b]	Mol. wt.	pI[c]	Mobility at pH 8.6[d]	Other distinctive characteristics
α-Casein	Casein	45–63	?	4.1	−6.7	1% Phosphorus
β-Casein	Casein	19–28	24,100[e]	4.5	−3.1	0.6% Phosphorus
γ-Casein	Casein	3–7	?	5.8–6.0	−2.0	0.11% Phosphorus
β-Lactoglobulin[f,g]	Lactalbumin	7–12	35,500	5.18	−5.1	1.1% Cysteine
α-Lactalbumin[f]	Lactalbumin	2–5	15,100	4.1–4.8	−4.2[h]	7% Tryptophan
"Blood" serum albumin[i]	Lactalbumin	0.7–1.3	65,000	4.7	−6.7	Appears to be identical to albumin of bovine blood
Immune globulins:						
Euglobulin	Lactoglobulin	0.8–1.7	180,000[j]	6.0	−1.8	Carry antibodies. Present in high concentration in colostrum. Contain hexose and hexosamine.
Pseudoglobulin	Lactoglobulin	0.6–1.4	180,000[j]	5.6	−2.0 to −2.2	

[a] From Jenness *et al.* (119).

[b] As determined by measurements of relative areas of electrophoretic components in casein (1.5%) and serum protein (2.0%) fractions at pH 8.6 and ionic strength 0.1.

[c] Isoelectric point, i.e. pH of no electrophoretic migration.

[d] Electrophoretic mobility in units of 10^{-5} cm.^{2} volts^{-1} sec^{-1} obtained by the Tiselius moving boundary method at ∼2° C. in veronal buffer at pH 8.6, ionic strength = 0.1, (0.02 *M* veronal, 0.1 *M* Na veronal).

[e] Value applies below 15° C. only.

[f] Prefixes α and β for α-lactalbumin and β-lactoglobulin were assigned on the basis that these proteins sediment at the same rates in the ultracentrifuge as peaks designated α- and β- for skim milk by Pedersen.

[g] β-Lactoglobulin appears to consist of two closely related components designated β_1 and β_2 lactoglobulin which differ slightly.

[h] Mobility −3.6 in the milk serum protein mixture.

[i] Some of the data for this protein are for material actually isolated from blood.

[j] Protein not entirely homogeneous; molecular weight of major component.

(mobilities 2.9 and 4.6) were composed of water-soluble and water-insoluble fractions. Although these components have mobilities similar to α- and β-globulins of serum, the water-insoluble portions were immunochemically different. The water-soluble fractions were, however, precipitated by the serum of rabbits immunized with human serum. Component 4 (mobility 5.7) was found to be a glycoprotein containing 9.3% nitrogen and 26.8% reducing substance (as glucose) following treatment with $1\ N$ HCl at 100° C. It contained 10.8% hexosamine and no uronic acid. The fifth component was probably a water-soluble modification of the fourth, because it does not appear in fresh plasma.

Serum albumin and γ-globulin appear to be essentially absent in seminal plasma, although traces of albumin can be demonstrated serologically.

Plasmas from patients whose seminal fluids were abnormally viscous or where the sperm were either poorly motile or nonmotile or which contained no sperm were not significantly different from normal specimens (Fig. 31) (122). The variations in the so-called "normals" were as great as in the abnormals.

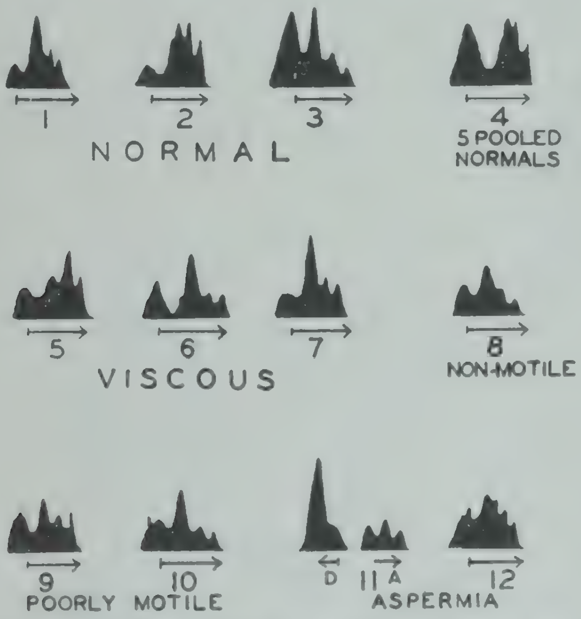
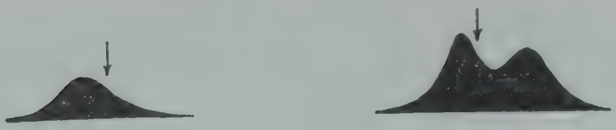


FIG. 31. Patterns of seminal plasma in phosphate-saline buffer, pH 7.85, ionic strength 0.1. Only ascending patterns are shown except in No. 11 where there is a marked discrepancy between ascending and descending limbs (122).

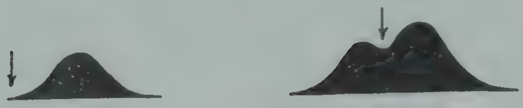
G. Hemoglobin

In no field of application has electrophoresis been more decisively helpful than in the study of hemoglobins. At present 10 different types of



a) Normal

c) Sickle Cell Trait



b) Sickle Cell Anemia

d) 50-50 Mixture of a) and b)

FIG. 32. Patterns of carbonmonoxyhemoglobins; phosphate buffer, pH 6.90, ionic strength 0.1 (123).

hemoglobin in human subjects have been described. Differences in electrophoretic properties were first noted in cases of sickle-cell anemia (123). About 0.8% of Americans have erythrocytes which reversibly change their shape from biconcave disks to a crescent or sickle shape upon reduction of the available oxygen, and about 2% of these individuals suffer from a severe chronic anemia. The people who have this trait are said to have sicklemia and those who have excessive destruction of the sickling cells are said to have sickle-cell anemia. The sickling process was found to be due to the presence of a pathologic hemoglobin which in either the carbon-

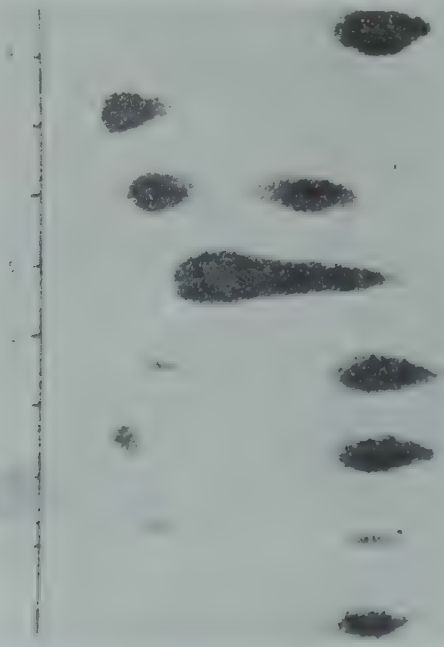


FIG. 33. Paper electrophoresis of a number of hemoglobin specimens containing various types of hemoglobin. Numbers 2 and 4 are examples of hemoglobin E trait and No. 6 is an example of Mediterranean hemoglobin E disease (125).

monoxy or ferro form has an isoelectric point at pH 7.09, whereas in the same phosphate buffer ($\mu = 0.1$) the normal hemoglobin is isoelectric at pH 6.87. Electrophoretic differences in the two hemoglobins are illustrated in Fig. 32. People with the sickle-cell trait or sicklemia have erythrocytes containing 20 to 45% of this hemoglobin S and those with sickle-cell anemia have 80 to 100%. The basic cause for the difference in these hemoglobins is genetic.

It was not long until another type of hemoglobin was identified by means of electrophoresis and denaturation (124). By exposing hemoglobin solution to an alkaline reagent at pH 12.7 it was found that human fetal hemoglobin was resistant to denaturation. It has distinctive electrophoretic properties, as is demonstrated in the paper electrophorogram of Fig. 33 (125). This fetal variant, hemoglobin F, usually disappears during the first year or two of postnatal life, but occasionally it has been demonstrated in adult blood. Another type, found in Americans with a distinct hemolytic syndrome which is intermediate between the benign sickle-cell trait and sickle-cell anemia, is hemoglobin C (originally referred to as hemoglobin III, hemoglobin c or hemoglobin X). It is often found in combination with hemoglobin S (Fig. 34) (126, 127).

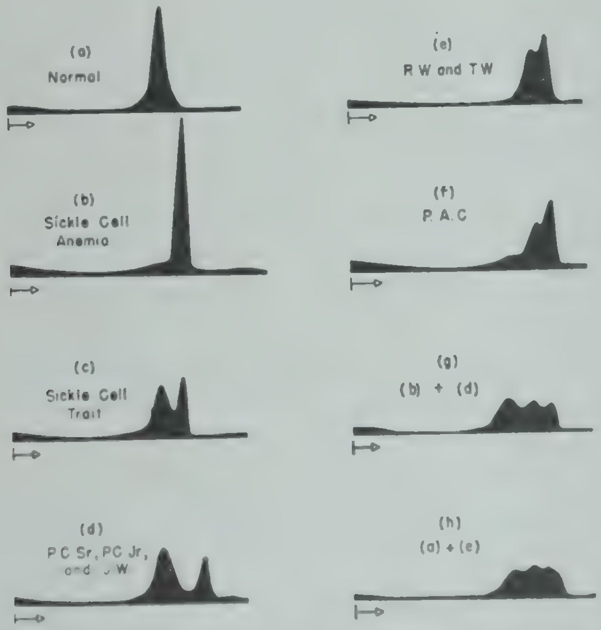


FIG. 34. Patterns of hemoglobins at pH 8.6. (a) Hemoglobin A only; (b) hemoglobin S with a small amount of hemoglobin F; (c) hemoglobins A and S; (d) hemoglobins A and C in hemoglobin C trait; (e) hemoglobins S and C in sickle-cell-hemoglobin C disease; (f) hemoglobins F, S, and C in sickle-cell-hemoglobin C disease; (g) and (h) mixture experiments to demonstrate differences in mobility among hemoglobins A, S, and C. The initials in (d), (e), and (f) refer to the subjects having these patterns (126).

Hemoglobin E is associated with Mediterranean anemia. Hemoglobin H (128) was found in a family living in Oregon, and hemoglobin I (129) and J (130) were found in other families. These hemoglobins in veronal buffer at pH 8.6 all have mobilities greater than normal adult hemoglobin A. Hemoglobin A has the highest isoelectric pH followed by J, I, and H, respectively.

H. Muscle Extracts

Extracts of minced rabbit muscle have been extensively studied by Jacob at various pH values and ionic strengths (131). As many as eleven components have been described in such extracts. Mobility-pH curves for eight of these components are given in Fig. 35. At all pH values three groups of constituents are apparent. Group I, which accounts on the average for 72% of the refractive material, consists of components l, m, and n with isoelectric points between pH 6.0 and 6.75. Group II (components j and k), representing 16.8% of the pattern, is isoelectric at pH 5.0 to 5.5. Group III, representing 5% to 8.6%, has a lower isoelectric range. A pattern of an extract analyzed at pH 6.6 is reproduced in Fig. 36. The Group I proteins seemed to be composed primarily of myogen. Myosin probably

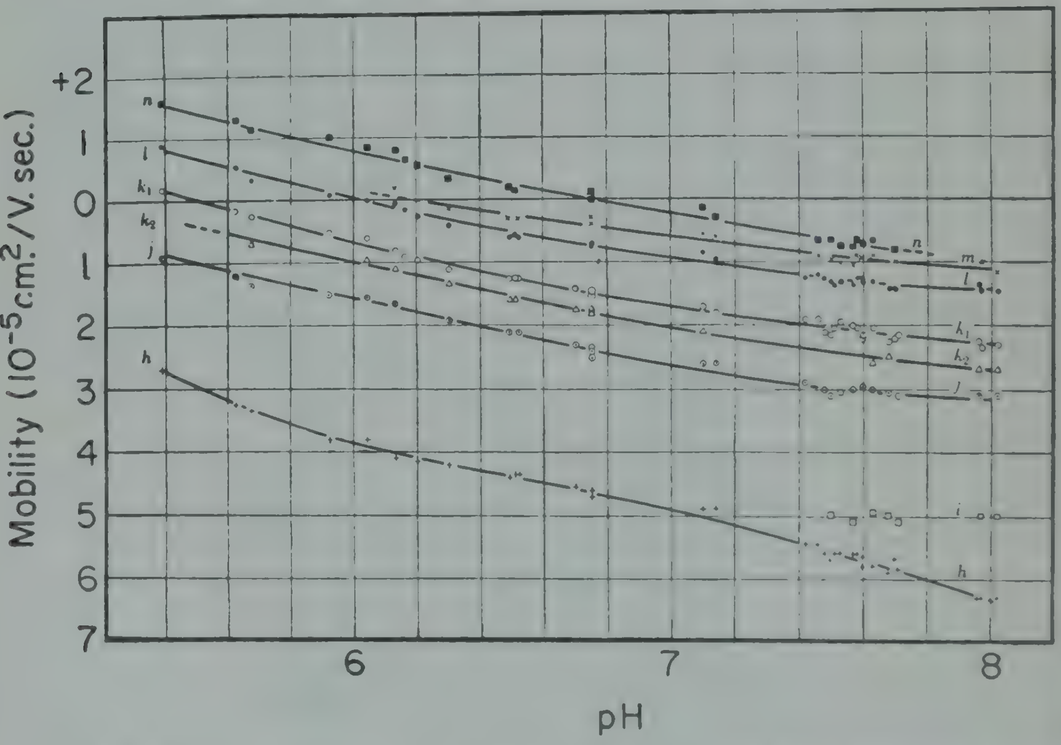


FIG. 35. Electrophoretic pattern of extract from perfused skeletal muscle of rabbit. Phosphate buffer, pH 6.6, ionic strength 0.15 (131).

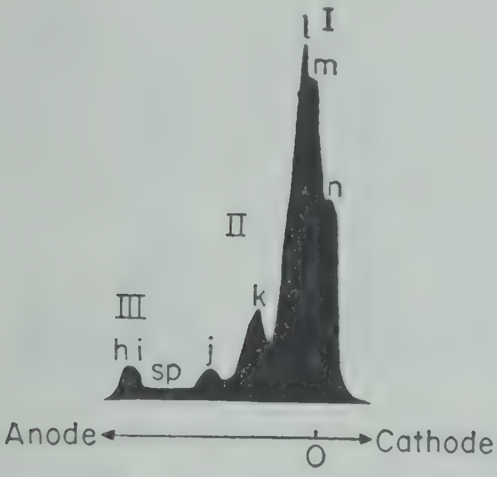


FIG. 36. Mobility-pH curves for the chief constituents of rabbit skeletal muscle at ionic strength 0.15 (phosphate buffer containing 0.05 *M* NaCl) (131).

falls in group II and myoalbumin in group III, the named proteins being more complex than was originally thought. Bondarenko has described four components of rabbit muscle extract. The identification and relative concentration of these fractions were as follows: albumin 6.1%; α-myosin, 47.0%; β-myosin, 20.3%; and myogin, 25.1% (132).

Phosphorylase was found by Toschi (133) to be associated with component k of Jacob's group II, aldolase and triphosphate dehydrogenase with components l and m of group I and α- and β-adenosinetriphosphatase with two components probably falling in group II (133). Myosin prepared by the method of Greenstein and Edsall (134) and adenosinetriphosphatase activity could not be separated by electrophoresis (135).

Amberson *et al.* (136) have studied muscle extracts obtained by soaking whole muscles for a prolonged period of time in 0.4 *M* potassium phosphate buffer at pH 7.6. Two or three electrophoretic components were usually obtained by this method. By adding 500 mg. per cent of sodium pyrophosphate to the buffer, much more protein and another large component were extracted.

Mouse and dog leg muscle proteins extracted with 0.9% NaCl have been studied rather extensively in an attempt to understand the effect of injuries such as ischemia (tourniquet) and scald (137–139). A species difference in the patterns was found, as has also been noted by Dubuisson (140) and Nikkila and Linko (141). After tourniquet injury the component with the mobility of albumin (Jacob's component h), which was normally very small, became the predominant component in the pattern and the Group I proteins (myogen) were greatly reduced (139). The high concentration of proteolytic or autolytic enzymes in muscle tissue makes necrosis

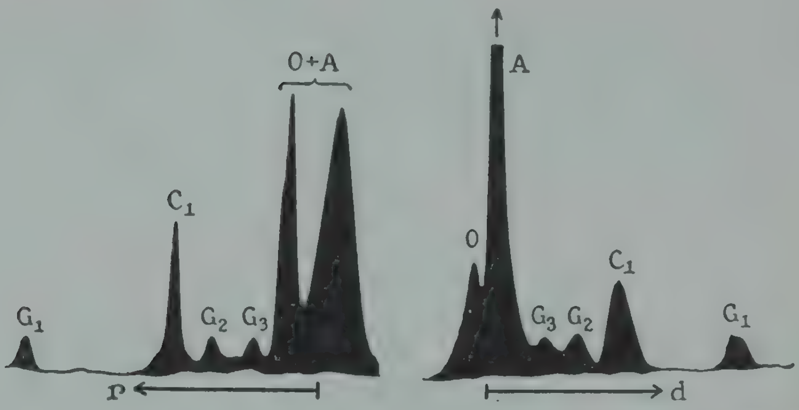


FIG. 37. Ascending and descending patterns of egg white, diluted three times, in a 0.1 N sodium acetate buffer at pH 4.5 (142).

an important factor when determining the nature of the protein pattern of an extract. In injury the muscle proteins appear to undergo considerable autolysis and are largely replaced by plasma proteins, particularly plasma albumin.

I. Egg White

A thorough investigation of the proteins of egg white has been carried out by Longsworth and co-workers (142). By employing a combination of chemical methods and electrophoretic analyses over a wide pH range eight distinct components were identified at the following relative concentrations: ovalbumin, A_1–A_2, 60%; conalbumin, C_1–C_2, 14%; ovomucoid, O.

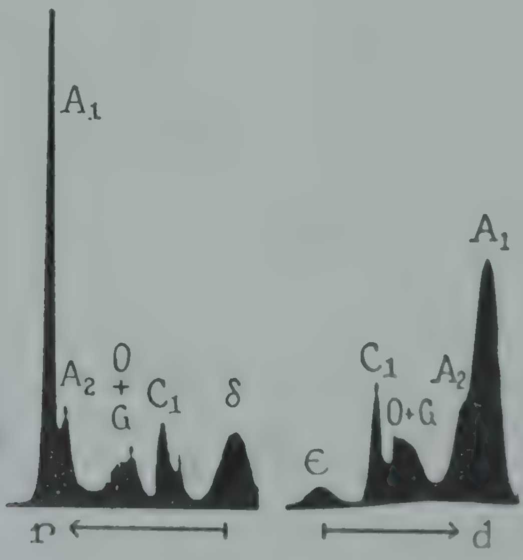


FIG. 38. Ascending and descending patterns of egg white, diluted eight times in a phosphate buffer at pH 8.0, ionic strength 0.1 (142)

14% globulins, G_1, 2.8%, G_2, 1.6%, G_3, 4.3%. A pattern of whole egg white (chicken) at pH 4.5 is shown in Fig. 37, and at pH 8.0 in Fig. 38. The two components of albumin are resolved only between pH values 5.0 and 10.0, and other components are unresolved at several pH values as may be seen in pH-mobility curves of Fig. 39. The dual nature of ovomucoid is not clearly resolved at any pH value, but its complexity is indicated by reversible boundary spreading, i.e., the broad boundary tends to resharpen as it returns toward its starting position under the influence of a reversed electric field.

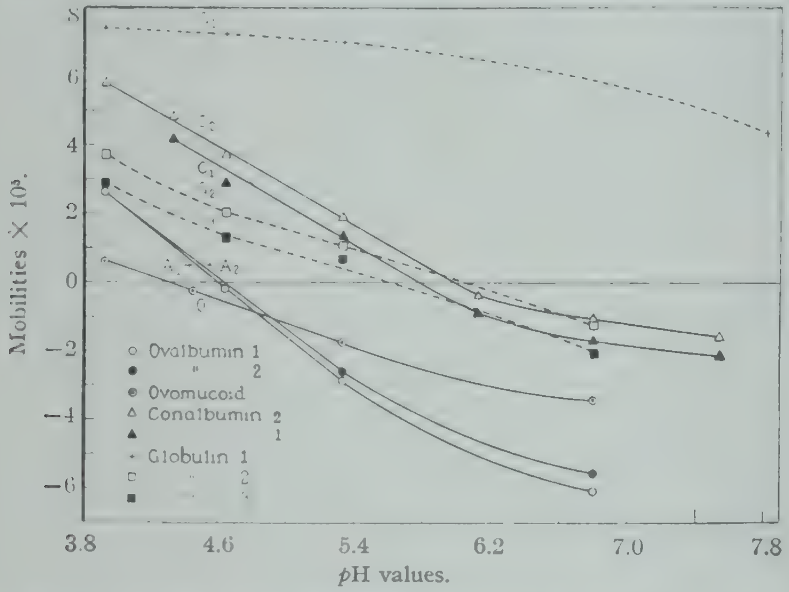


FIG. 39. Electrophoretic mobilities of the constituents of egg white as functions of pH (142).

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Chapter 10

The Electrophoresis of Viruses, Bacteria, and Cells, and the Microscope Method of Electrophoresis[1]

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[1] Publication no. 55 of the Department of Biophysics, University of Pittsburgh, Pittsburgh, Pennsylvania.

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I. INTRODUCTION

The region of a biological particle, whether it be virus, bacterium, or cell, which is accessible to electrophoretic study is its surface. Knowledge of the surface is extremely useful because, far from being simply an interface between the particle and its environment, the surface is the site of many biological phenomena of importance.

The motion of a particle in solution caused by an externally applied electric field is expressed experimentally as the electrophoretic mobility, u. From theoretical considerations two other parameters can be calculated: the potential difference between the surface and the solution, ζ, and the average net surface charge density, σ. σ depends on the composition of the surface itself and on the ionic constitution of the medium; ζ depends on σ, ionic constitution, surface contour, and dielectric constant of the medium; u depends on σ, ionic constitution, surface contour, and viscosity of the medium. Therefore, in order to obtain information about the composition of the surface itself, one would like to know the value of σ. Unfortunately, it is difficult to obtain an accurate estimate of σ for biological particles because knowledge of the parameters necessary to calculate σ from the mobility is usually inadequate. However, many useful applications of the electrophoretic method involve only separation of differently charged particles, and for this a detailed knowledge of the theory is inessential.

The variation of electrophoretic mobility among different particles depends upon the fact that the chief constituents of biological material (proteins, lipids, polysaccharides, and nucleic acids) have quite different electric charge behaviors. The number of different combinations of these constituents on biological surfaces is, of course, enormous, but the surface composition of a population of the same kind of particles is amazingly uniform. It is upon these two facts that the usefulness of electrophoresis depends.

The aims of this chapter are to examine the features of existing electrophoretic theory that are of particular importance to the study of the larger biological particles, i.e. viruses, bacteria, and other cells; to describe the microscope method of electrophoresis; to indicate the types of information which can be obtained by the electrophoretic method; and to present as comprehensive a review of the important research results as space and time will allow. Throughout the chapter an effort has been made to distinguish between what is theoretical inference and what is experimental fact. Special attention has been given to the limitations of the experimental technique and to the pitfalls of mobility data interpretation.

Several review articles and books (1–10) may be consulted for further details and for additional references, especially to the older literature.

II. Theory and Calculations for Aqueous Suspensions of Viruses and Cells

A. Origin and Distribution of Particle Charge

Particles in solution acquire a charge because of ionization of their surface and adsorption of ions. Because most of the ionizing groups are weak electrolytes and because binding energies for adsorption are of the order of magnitude of thermal energies, the surface charge situation must be described as an equilibrium between ions bound to the surface and ions in solution.

A thermodynamic equilibrium implies that the charge might fluctuate as a function of time. However, the fluctuations occur in a much shorter time than that required to measure particle mobility and, therefore, a statistical time average of the surface charge will be obtained by electrophoresis. This observation might seem superfluous; however, surface charge phenomena could exist (adsorption of a virus to a cell surface, for instance) which might depend upon a local arrangement of charges that would be expected to fluctuate with time. No local patterns of charge or their fluctuations can be detected by mobility measurements.

Ionizable groups on the particle surface become charged by the gain or loss of hydrogen ions and other ions. Hydrogen ions receive special attention because their concentration is easily controlled and measured and they readily combine with biological substrates, for example, the amino, carboxyl, and other groups of proteins. The pH dependence of the surface charge density and the mobility are usually different for different substances.

The forces involved in preferential adsorption of ions to the surface are essentially electrostatic in nature, the ions being attracted by ionized groups, induced electric dipoles, and hydrogen bonds. In addition to binding ions, many substances can bind water; the greater the amount of water bound to the surface, the less the ion adsorption. The hydration of the ion is important for the same reason; negative ions, being less hydrated than positive ones, are more strongly bound. Another way of looking at the same question is that the affinity of water for positive ions is greater than for negative ions, so that negative ions tend to be excluded from the bulk of the liquid and to accumulate at interfaces. Gas bubbles, for instance, acquire a negative charge (11). The greater polarizability and smaller size of negative ions also contribute to their preferential adsorbability.

A simplified view of the charge origin of biological substances is that the charge on proteins is mainly determined by their ionizable groups, adsorption being limited by hydration. The charge on lipids is mainly due to adsorption, because they are unhydrated and possess fewer ionizable groups. The charge on polysaccharides is small because they are hydrated and have few ionizable groups. The surface charge behavior of viruses is the same as that of proteins; the surface charge characteristics of cells can correspond with any one or a combination of the behaviors of proteins, lipids, or polysaccharides. Particles can become nonspecifically charged by the exclusion of negative ions from the bulk of the liquid and their resultant accumulation at interfaces. This effect is most important for hydrophobic surfaces.

A special case of ion-binding is that of chemical interaction, for example, the binding of picrate ion by protein (12). Because of the greater binding energy of covalent chemical bonds compared to electrostatic-type bonds, the ion concentration in this case will have a much greater effect on the surface charge.

B. Origin and Distribution of Counterion Charge

In the liquid surrounding the charged particle, there is an excess of ions of opposite sign of the net surface charge. The distribution of the resulting volume net charge density can be calculated from a consideration of the equilibrium between electrical energies and thermal energies. The results of such calculations for a given particle shape give the electrical potential, Ψ, as a function of the distance from the particle surface, x. If the Debye-Hückel approximation holds, the solution for a flat, smooth surface is

$$\Psi = \zeta e^{-\kappa x} \tag{1}$$

where κ is the familiar Debye-Hückel constant, and ζ is the potential at the particle surface with respect to the bulk of the liquid.

C. Equations of Particle Motion

From a consideration of the charge distribution in the double layer and the law of viscous flow of liquids, one form of the electrophoretic equation of motion, the Helmholtz-Smoluchowski equation, can be derived.

$$u = \frac{\zeta D}{4\pi\eta} \tag{2}$$

u = electrophoretic mobility = velocity per unit field;
ζ = potential difference between the surface of shear and the bulk of the liquid;
D = dielectric constant of the suspending medium;
η = viscosity of the suspending medium.

This equation shows that mobility is proportional to the electrokinetic or zeta potential and is only indirectly connected with the surface charge. Several important assumptions are involved in its derivation.

(1) The product of the Debye-Hückel constant, κ, and the radius of curvature of the particle surface is large.

(2) The particle surface is nonconducting.

(3) The conductivity, dielectric constant, and viscosity have the same values within the double layer as in the bulk of the liquid.

(4) The applied electric field, although distorted by the presence of the particle, can be simply added to the field of the double layer.

(5) The liquid at the particle surface has the velocity of the particle, and the velocity gradient begins at the surface.

The validity of these assumptions is discussed in detail in the chapter on theory. However, some of the implications of the equation and the assumptions involved in its derivation are of special importance for viruses and cells.

For most biological surfaces, assumption (5) is problematical. However, the particle surface may be defined in such a way that the assumption holds by postulating a "surface of shear," which is the surface at which the liquid is stationary with respect to the particle. The surface of shear might not coincide with the particle surface as defined by microscopy or hydrodynamic methods. For a hydrated particle, for instance, it could be outside the actual particle surface.

For biological particles, assumption (3) might be invalid under certain conditions. Water can form a crystalline structure around hydrophobic surfaces (13) and is subject to electrostriction in the neighborhood of charged centers on proteins. Assumption (3) might also be invalidated by the presence of metabolic products diffusing away from a cell. Washing the cell before measuring mobility helps to eliminate this objection but might also remove characteristic surface structures.

The validity of assumption (4) is thoroughly discussed by Overbeek (7).

Assumptions (1) and (2) are made so that the electric field can be considered parallel to the surface everywhere within the double layer. If, on the other hand, the field is considered to be everywhere in the direction of the applied field, a different equation is obtained (14):

$$u = \frac{\zeta D}{6\pi\eta} \text{ (for spheres)} \tag{3}$$

This equation derived by Debye and Hückel is valid when κa is small, where a is the radius of curvature of the surface. Henry (15) has derived an equation valid for any value of κa, which can be expressed in the form,

$$u = \frac{\zeta D}{6\pi\eta} f(\kappa a) \tag{4}$$

where $f(\kappa a)$ depends upon the shape, size, and orientation of the particle. For κa less than 100, or greater than 1/10, Henry's correction becomes appreciable. In Fig. 1 the radius for $\kappa a = 100$ and $\kappa a = 1/10$ is plotted against concentration of a uni-univalent (1–1) electrolyte. It is obvious from the figure that Smoluchowski's equation would be valid for bacteria and cells at usual salt concentrations if a was the radius of the whole particle. However, if a is taken to be the radius of curvature of the particle surface, Henry's equation must be used even for bacteria and cells. For viruses at most salt concentrations, Henry's correction must be applied. The function $f(\kappa a)$ in equation (4) varies between 1 and $\frac{3}{2}$ and is tabulated in Abramson *et al.* (4) and in Overbeek (7). As written here it is equal to of the original factor as proposed by Henry (15).

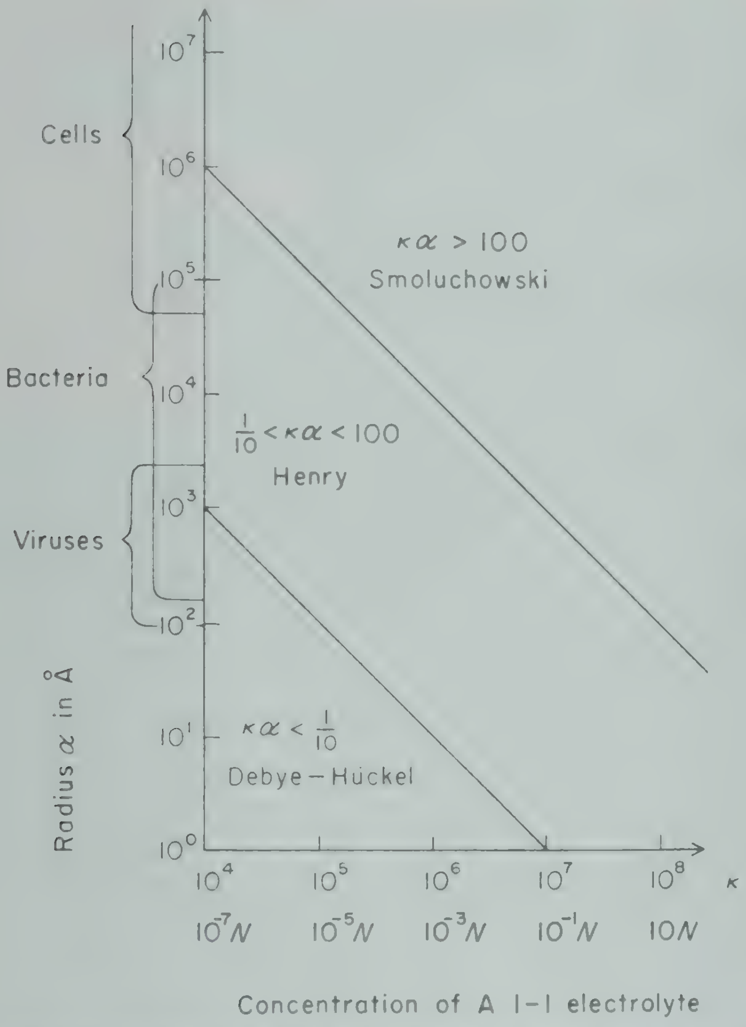

FIG. 1. The product κa plotted as a function of κ and a to show the regions where the equations of Smoluchowski, Henry, and Debye-Hückel are applicable.

D. Effect of Particle Size and Shape on Mobility

1. Large, Smooth Particles ($\kappa a > 100$)

The derivation of equation (2) is independent of the size and shape of the particle. A large amount of experimental evidence (16, 17) showing that the mobility of microscopically visible particles is independent of size, gross shape, and orientation over a wide range has been frequently invoked as evidence for the universal applicability of equation (2) to large biological particles. However, it must be noted that, if the detailed surface contour of the particle is the mobility-determining shape factor rather than gross dimensions, one would not expect the mobility to vary with gross size and shape and the above evidence could not be considered as validating the Helmholtz-Smoluchowski equation. Evidence in favor of the above hypothesis is presented below. The case of large, smooth particles, where $\kappa a > 100$, almost never exists for viruses and cells, if a is considered to be the radius of curvature of the surface.

2. *Small Particles* ($\kappa a < 100$)

Since $f(\kappa a)$ in equation (4) depends upon particle size and shape, it is interesting to examine particle suspensions, heterogeneous with respect to size or shape as determined in the ultracentrifuge or electron microscope, for heterogeneity with respect to electrophoretic mobility. Eriksson-Quensel and Svedberg (18) in a precise analysis of the size distribution and mobility of tobacco mosaic virus in 0.02 M buffer ($a = 8$ mμ, $\kappa a = 3.7$) found that the virus migrated very uniformly in the electric field in spite of large inhomogeneities with respect to molecular weight. Bacterial flagella (19), prepared by shaking and having a considerable length distribution, migrated with a single very sharp boundary in 0.1 ionic strength buffers ($a = 6$ mμ, $\kappa a = 6.2$). A preparation of bacterial capsular filaments (20), also prepared by shaking, having a ratio of longest particle to shortest particle of greater than 50:1 migrated with a single sharp peak in 0.02 ionic strength buffer ($a = 5$ mμ, $\kappa a = 2.3$). An ultracentrifugal analysis showed an extremely diffuse boundary after only a few minutes of sedimentation. All the above suspensions can be considered to consist of cylinders of varying lengths but with constant radii. Since the radius rather than the length of cylinders determines the amount of the Henry correction, a heterogeneity in cylinder length should not result in a heterogeneity in mobility.

Henry's equation predicts different mobilities for spheres of different radii for the region $1 < \kappa a < 100$. Unfortunately, there are very few quantitative data in this region to confirm or deny Henry's theory. Quantitative data do exist for the mobility of free protein molecules compared to the mobility of microscopically visible particles coated with the same protein. In many cases the two mobilities are nearly identical (4), although for free protein molecules at usual salt concentrations κa is small and for microscopically visible particles κa is large. According to theory, for large κa the mobility of spheres should be several times the value for small κa if surface charge density and all other conditions remain constant. That the mobilities are identical is possibly due to the choice of the radius of curvature a. If the large-particle surface is composed of many protein molecules maintaining their separate identities sufficiently so that the effective radius of curvature of the protein-coated particle is that of the protein molecule rather than that of the particle, theory does not conflict with experiment.

The only information available about the contour of large biological particles comes from electron micrographs, which show that surface structures with small radii of curvature are indeed commonplace on bacteria and cells. Bacteria can have several kinds of filamentous appendages including flagella ($a \cong 60$ Å.), and capsular filaments ($a \cong 40$ Å.) (Fig. 2). The bacterial cell wall shows an apparent fine structure in electron micrographs (21, 22). The red blood cell surface can have a fine structure as indicated by electron microscope data (23) or as postulated to explain virus hemagglutination (24). For flagella or capsular filaments of known radius, the calculation of bacterial ζ potential can be made correctly using

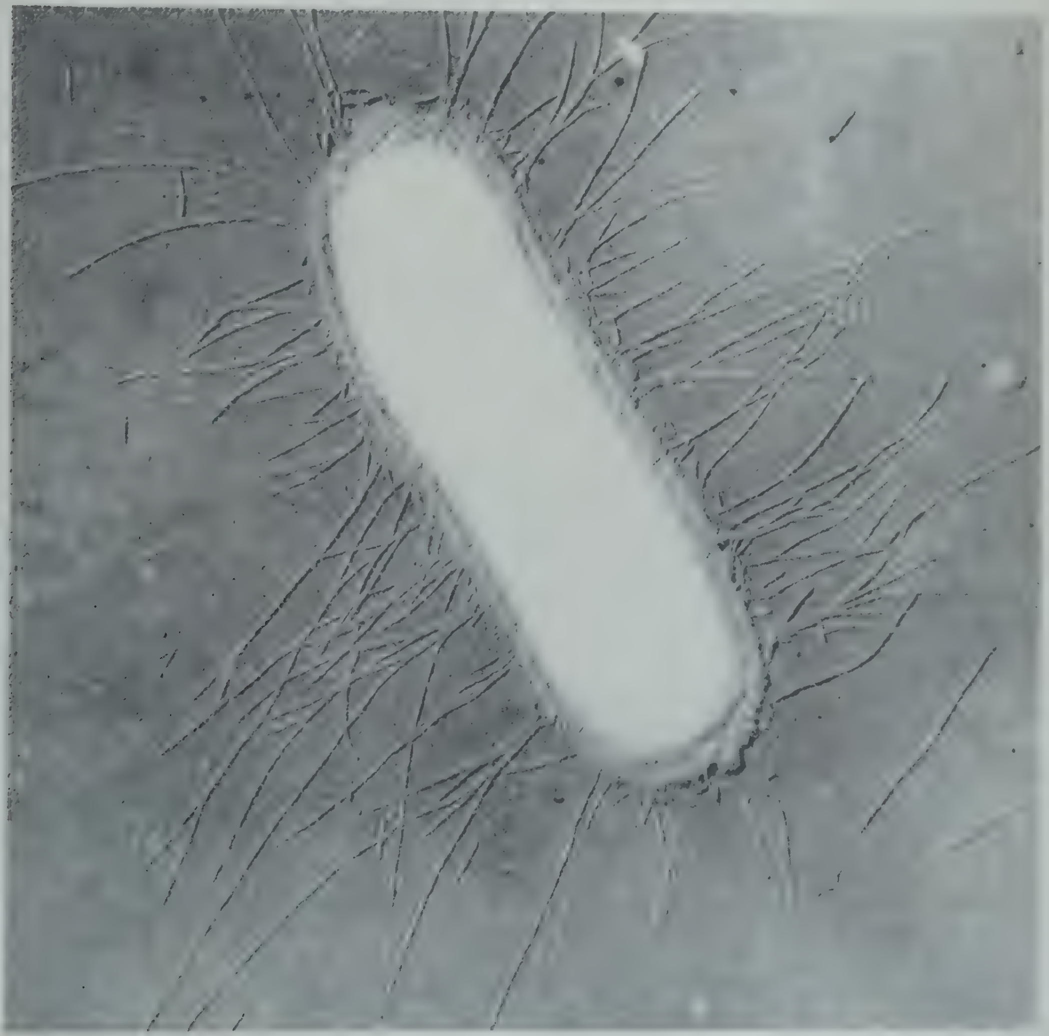

FIG. 2. Electron micrograph of a strain of *Escherichia coli* S showing capsular filaments about 80 Å. in diameter. The R form of the same organism has no filaments. Photograph taken by C. C. Brinton, Jr., at the Biophysics Department of the Institute of Physics in Geneva, Switzerland, through the courtesy of Dr. Edouard Kellenberger.

Henry's equation as long as most of the particle mobility is due to the flagella or filaments. An electron microscopic examination of the bacterium or cell in question is necessary for an unequivocal interpretation of electrophoretic data. It is doubtful that the simple Helmholtz-Smoluchowski equation is ever valid for biological particles at the salt concentrations practicable for mobility measurements.

E. Calculation of the Average Net Surface Charge Density

For spherical particles,[2] and for small enough ζ potentials so that the Debye-Hückel approximation $\nabla^2\Psi = \kappa^2\Psi$ is valid, the charge density is given by

$$\sigma = \frac{D\zeta}{4\pi}\frac{1 + \kappa a}{a} \tag{5}$$

For large, smooth particles ($\kappa a > 100$) this becomes

$$\sigma = \frac{D\zeta\kappa}{4\pi} \tag{6}$$

Substituting the value of ζ from equation (2) into equation (6), one obtains the simplest form of the expression for mobility in terms of charge density, valid for large smooth particles:

$$u = \frac{\sigma}{\eta\kappa} \tag{7}$$

For large values of ζ and polyvalent electrolytes, the Debye-Hückel approximation is no longer valid and a solution to the more exact equation must be found. A solution in closed form is available only for large κa (4):

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}}\sqrt{\Sigma c_i[\exp(-z_i e\zeta/kT) - 1]} \tag{8}$$

where c_i represents molar concentrations and z_i, charges of the ions. This equation, in combination with equation (2), has been used by most investigators for the calculation of the average net surface charge density of bacteria and cells. Because the value of σ is very sensitive to changes in a, the radius of curvature, errors approaching an order of magnitude can be made in σ if a is considered to be very large when it is actually much smaller. The correction factor for the ζ potential is not as sensitive to changes in a; the maximum error in ζ caused by overestimating a is 50%.

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[2] For particles other than spheres, where $\kappa a < 100$, other equations must be used. The only other shapes for which solutions exist are cylinders and flat disks (4).

Equation (4) for the mobility can also be corrected for the deformation of the double layer due to the applied electric field (7). Equations (5) and (6), in addition to the correction for higher ζ potentials given by equation (8), can be corrected for the finite size of the ions in the double layer (4). In general, these corrections increase with increasing values of ζ potential and κa. Under the conditions most frequently encountered in electrophoresis of bacteria and cells, these corrections are small compared to the uncertainties arising because of inexact knowledge of the radius of curvature.

An illustration of the errors in ζ and σ which can arise from incorrectly estimating a can be found in the case of filamented bacteria (Fig. 2) (24). Two kinds of cells exist, those covered with filaments and those completely devoid of filaments. The mobility of the two forms is different and the difference has been shown to be due to the presence of filaments, since their mechanical removal increases the mobility of the bacterium to the same value as that of the form devoid of filaments. The filamented bacteria form smooth colonies (S form) while the nonfilamented kind form rough colonies (R form). Mobility-salt concentration curves for the R and S forms have been obtained (20) and are quantitatively very similar to curves of the same type obtained by Moyer (25), using the same buffer for another strain of *Escherichia coli* R and S. It is very likely that the mobility difference observed by Moyer was also due to filaments. For filamented bacteria, the radius of curvature of the surface is known within ±20% to be 40 Å and the mobility-determining surface contour is made up of cylinders oriented randomly with respect to the external field. Henry's equation permits the calculation of the ζ potential for such a shape and orientation. Knowing the ζ potential, one can calculate σ from an equation derived by Gorin (Abramson *et al.*, 4):

$$\sigma = \frac{D\zeta}{4\pi a} \kappa a \frac{K_1(\kappa a)}{K_0(\kappa a)} \tag{9}$$

where the length of the cylinder is great compared to its radius, and K_1 and K_0 are Bessel functions. Sigma, as calculated from Moyer's mobility data for *Escherichia coli* S using the above equations, is compared in Fig. 3 with sigma as calculated by Moyer assuming the bacterium to have a smooth surface. The sigma-concentration (σ-C) curve calculated from the theory using the corrected radius of curvature and shape factor is quite different, both in absolute value and general shape, from the curve calculated assuming $a = \infty$. It should be pointed out that the corrected curve does not fit a Langmuir adsorption isotherm.

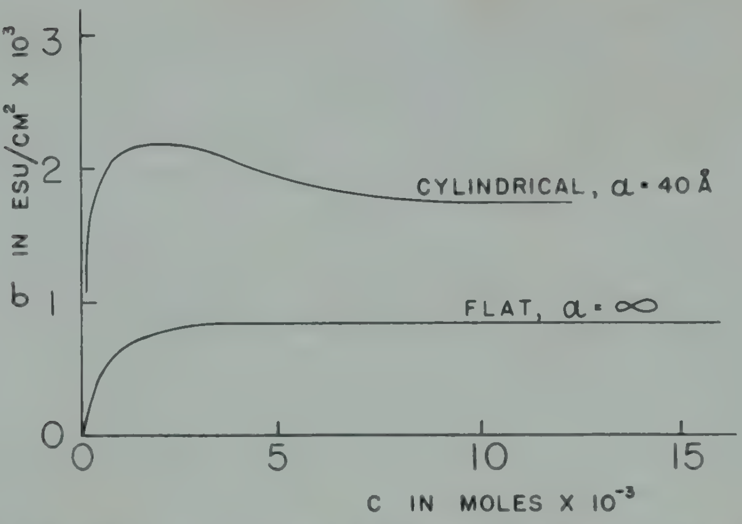

FIG. 3. σ-C curves for *Escherichia coli* S calculated from the data of Moyer (25), assuming either a flat, smooth surface [equations (2) and (8)] or a surface composed of randomly oriented cylinders [equations (4) and (9)].

F. Temperature Coefficient of Mobility

It is customary in considering the effect of temperature on mobility to assume that the charge density, σ, remains constant and that the change with temperature of the viscosity and dielectric constant of water controls the change in mobility. Equation (7) shows that, in the limiting case of large, smooth particles, u is proportional to $\sqrt{DT}$ and inversely proportional to η when σ is constant.

As temperature increases, there is a compensating decrease in the dielectric constant of water with the result that the term $\sqrt{DT}$ changes very little in comparison to the viscosity. Values of η and $\sqrt{DT}$ are shown in Table I for different temperatures. Because nearly all the effect of temperature on mobility is due to change in the viscosity of water, the following relationship can be used to correct the mobility to any temperature:

TABLE I

EFFECT OF TEMPERATURE ON THE DIELECTRIC CONSTANT AND VISCOSITY OF WATER

T (°C.)	η (centipoises)	D	$\sqrt{DT}$
0	1.79	88	15.5
20	1.00	80	15.3
100	0.28	48	13.4

$$\eta_1 u_1 = \eta_2 u_2 \qquad (9a)$$

Even when equations (5) or (6) are used for σ and Henry's equation [equation (4)] is used for ζ, equation (9a) still holds within a few per cent as long as ζ is not too large. This relationship has been verified experimentally for mineral oil droplets and paraffin wax particles in water (26) and for silver sol particles (27). However, horse serum albumin has a mobility difference of 10% when measured at 0° and 25° C. at pH 7.7 (28) even after the temperature correction has been applied. This difference is attributed to a difference in the charge density at the two temperatures. Equation (9a) should be applied cautiously over wide temperature ranges.

Field strength in electrophoresis is determined by the current, the cross section of the cell, and the specific conductivity of the buffer. Because conductivity increases with the temperature, the field strength will decrease if current is held constant. However, the increase in conductivity of salt solutions with temperature is nearly balanced by the decrease of water viscosity with temperature, with the result that electrophoretic velocity at constant current is essentially unaffected by temperature changes, at least within a range of about 10°. The calculated value of the mobility is the true mobility for the temperature at which the conductivity of the buffer is measured. One result of the temperature independence of velocity is that electrophoresis cells need be thermostatted only to remove excess heat in order to prevent convection and not for the accurate control of temperature per se.

III. The Microscope Method

A. *Description of the Method*

This method of electrophoresis involves the direct observation of microscopically visible particles as they migrate in the electric field. The particles are suspended in a suitable buffer solution and placed in a small flat or cylindrical transparent cell through which an electric current is passed. The time for a particle to cover a given distance as measured with a micrometer eyepiece is determined by the operator for each individual particle. From this the particle velocity is calculated, and from the current, conductivity of the buffer solution, and cross-sectional area of the cell, the electric field strength is determined. The experimental results are expressed in terms of mobility, or velocity per unit field strength, whose units can be expressed as (μ/sec.)/(volt/cm.) (used for bacteria and cells) or (cm./sec.)/(volt/cm.) (used for viruses and smaller molecules). It must be kept in mind that, when substituting mobility values into the equations for ζ and σ, the centimeter-gram-second electrostatic unit must be used. This unit is (cm./sec.)/(abvolt/cm.) = 300 (cm./sec.)/(volt/cm.)

B. Advantages and Disadvantages of the Method

Advantages of the method include the following points:

(a) It is the only way in which the mobility of large particles, such as microorganisms and cells, can be accurately measured.

(b) The environment in which the particles are migrating does not change during the observation and is the same for all particles. This desirable characteristic is not true for moving boundary electrophoresis in which pH, ionic strength, and particle concentration can vary considerably across the boundary.

(c) Mobilities can be measured at very low ionic strengths. In the moving boundary method, boundary anomalies are accentuated at low ionic strengths.

(d) The shape, size, and orientation of the particles can be observed directly during the measurement.

(e) Differences in relative mobilities can be observed directly by watching the particles move simultaneously.

(f) Only small concentrations of suspended particles are required for observation. This brings about the additional advantage that particle interaction, a complication of the moving boundary method, is avoided.

(g) The apparatus is inexpensive and easy to maintain.

(h) High magnifications can be employed, resulting in greater sensitivity.

(i) Isoelectric points of submicroscopic particles insoluble at their isoelectric points can be determined from the mobility of their microscopically visible aggregates.

The chief disadvantages are:

(a) When particles of submicroscopic size are to be investigated, it is necessary to adsorb them onto carriers such as collodion particles or finely ground glass of the proper size range. While it is the usual experience that the particle takes on the electrophoretic mobility characteristic of the adsorbate, this is not universally true.

(b) It is impossible to detect inhomogeneity when submicroscopic particles are adsorbed onto carriers of microscopic size.

(c) The microscope method is difficult to adapt for preparative purposes.

C. Apparatus

1. General

Microscope electrophoresis apparatuses have in common a cell into which a microscope can be focused, an electrode system, and provision for filling, emptying, and closing the system. They vary as to the shape (flat or cylindrical), size, and orientation (horizontal, lateral, or vertical) of the cell, the type of electrode, the manner of assembly, and the manipulation required to fill, empty, and close the cell.

When electrophoretic migration is to be observed, it is paramount that any mechanical displacement of the liquid in the cell not due to the electric field be prevented. Unwanted mechanical movement can arise from convection because of unequal temperatures due to heating of the liquid by the electric current or the microscope illuminator, air bubbles introduced into the cell during filling, gassing of the electrodes, vibration, external pressure differences across the ends of the cell communicated by inadequate sealing, and electroosmotic streaming.

Convection is prevented simply by employing low currents and by using a heat filter between the microscope illuminator and the cell. Since the field strength for a given current decreases with salt concentration, more current must be used at high salt concentrations to obtain a measurable velocity. Thus, convection places an upper limit on the salt concentrations which can be employed. Experience has shown this limiting concentration to be in the neighborhood of 0.1 *M* for cells about 0.5 mm. in thickness. Since heating is proportional to the current squared and field strength is proportional to the current, maintaining the same field strength by halving the current and doubling the resistivity will halve the heat loss.

Air bubbles are precluded by careful cleaning and filling of the apparatus. The cell is usually kept filled with liquid and new suspensions are introduced by the displacement of old ones.

Gassing of the electrodes is prevented by employing reversible electrodes such as copper-copper sulfate or mercury-mercuric nitrate (29). This also prevents the build-up of polarization potentials which cause the current to fluctuate. The electrodes must be isolated in such a way that none of their electrolyte is washed into the cell in the filling process and no pressure differences are transmitted to the cell.

Vibration is eliminated by solidly fixing the cell to the stage of the microscope.

External pressure differences are prevented by closing the cell at both ends.

2. *Electroosmosis Within the Cell*

Electroosmotic streaming of the liquid past the cell walls cannot be eliminated or counteracted by any known method and must be taken into account if true electrophoretic velocity is to be measured. Since the system is closed, and, therefore, the net flow of liquid through the cell must be zero, the flow of liquid along the walls is counterbalanced by a flow in the opposite direction in the center of the cell. Therefore, the velocity of the liquid varies with the depth of the cell. This is true for both flat and cylindrical cells (including the U-tubes used in the Tiselius method) but the way in which the velocity varies is different in the two cases.

The variation of liquid velocity with cell depth is easily determined by classical hydrodynamic methods. For the purpose of this derivation we shall assume a flat cell whose width is large compared to its thickness.

Viscosity is the resistance encountered by one part of the liquid moving across another part of the same liquid. The unit of viscosity is defined such that unit force is required to cause two parallel liquid planes of unit area and at unit distance apart to slide past one another with unit velocity.

$$F = \frac{\eta A \Delta V_w}{h} \tag{10}$$

where F is the force, A is the area of each liquid plane, h is the distance between them, ΔV_w is the relative velocity of the two liquid planes, and η is the viscosity coefficient. Equation (10) can be expressed in differential form:

$$F = \eta A \frac{dV_w}{dh} \tag{11}$$

The symbol, V_w, now means simply the velocity at some level, h. In a closed cell, there is a tendency for liquid to move due to electroosmosis. This will result in a pressure difference, P, between the two ends of the cell, which will, in turn, cause the liquid to flow in the reverse direction. If one focuses attention on a layer of liquid with length, l, width, w, and thickness, dh, it follows from equation (11) that the viscous drag (the difference between the forces on the top and on the bottom of the layer) is given by $lw\eta \frac{d}{dh}\left(\frac{dV_w}{dh}\right) dh$, where $lw = A$. This drag must be balanced by the electroosmotic pressure multiplied by the area of the end of the layer, $Pwdh$. By equating these two forces and performing obvious algebraic operations, one obtains

$$\frac{P}{l} = \eta \frac{d^2V_w}{dh^2} \tag{12}$$

The total thickness of the cell is assumed to be $2a$; i.e. h varies from $-a$ to $+a$. Since the total flow of liquid must be zero,

$$\int_{-a}^{+a} V_w dh = 0 \tag{13}$$

Integrating equation (12), we obtain the velocity of the liquid as a function of the distance, h:

$$V_w = \frac{P}{2\eta l} h^2 + Ah + B \tag{14}$$

where A and B are constants of integration. For the case in which h is measured from the center of the cell, symmetry requires that A be 0. At the boundaries, where $h = \pm a$, the velocity of the liquid is equal to the electroosmotic velocity, U. By substituting this condition into equation (14), one obtains:

$$V_w = \frac{P}{2\eta l}(h^2 - a^2) + U \tag{15}$$

When equation (15) is substituted into equation (13), integrated over the distance $-a$ to $+a$ and solved for P, we find

$$P = \frac{3U\eta l}{a^2} \tag{16}$$

Upon substitution of equation (16) into equation (15) there results

$$V_w = \frac{U}{2}\left(\frac{3h^2}{a^2} - 1\right) \tag{17}$$

which gives the liquid velocity as a function of distance for a given electroosmotic velocity. This is the equation of a parabola symmetrical about the cell center and is plotted in Fig. 4. To find the levels where $V_w = 0$, we set

$$\frac{3h^2}{a^2} = 1 \quad \text{and} \quad h = \frac{a}{\sqrt{3}} = 0.578a$$

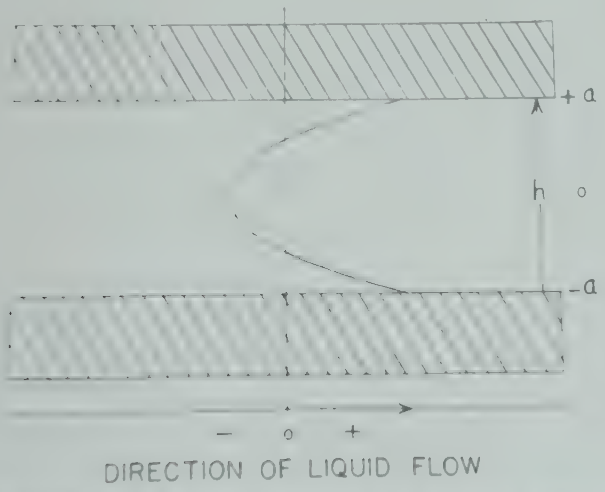

FIG. 4. Cross section of a flat electrophoresis cell showing magnitude and direction of liquid flow as a function of cell depth. The stationary levels, where the net flow of liquid is zero, occur at $h = \pm 0.58a$ for a cell whose width is large compared to its depth.

The stationary levels thus fall at 0.21 and 0.79 of the whole cell depth and are independent of the cell depth and the electroosmotic velocity. This result was first obtained by Smoluchowski (30) and holds for a cell whose width is great compared with its thickness. Komagata (31) studied the more general case of a cell whose width is not great compared to its thickness. If the ratio of cell width to thickness is taken as K and the position of the stationary layer in terms of the fractional depth from the top of the cell is a_0, Komagata's theory predicts that $a = \frac{1}{2} + \sqrt{\frac{1}{12} + 32/\pi^5K}$. This reduces to the solution derived above when $K = \infty$. For cells whose ratio width per thickness is greater than 40, Smoluchowski's theory still holds. For K less than 40, the values given in Table II should be used.

TABLE II

Position of the Upper Stationary Layer in Flat Cells as a Function of the Width : Thickness Ratio, K

K:	10	15	20	40	100	∞
a_0:	0.194	0.199	0.202	0.207	0.210	0.211

Since $V_{\text{obs}} = V + V_w$, where V_{obs} = observed velocity and V = velocity of the particle relative to the liquid, V can be most easily determined by measuring V_{obs} at the stationary levels where $V_w = 0$. According to equation (17), V could be found by measuring V_{obs} at any depth and subtracting V_w, which can be calculated if U is known. U can be determined experimentally for a given suspension and cell. Measuring V_{obs} at the center of the cell has the advantage that the velocity gradient there is zero and errors in focusing are, therefore, less important. It has the disadvantage that U is rather sensitive to the condition of the cell wall and might change from one observation to another. Nearly all accurate measurements of V recorded in the literature have been made at the stationary levels; errors in focusing are minimized by measuring velocities at both upper and lower levels and averaging.

The above theory and equations hold only for plane parallel cells whose two walls are composed of the same material. It is thus of prime importance to make sure a new cell is plane parallel; its thickness can be checked optically with the microscope fine-focus adjustment and should not vary more than a few per cent from side to side and end to end. One test for symmetry and flatness (32) is to coat the inside of the cell with gelatin and determine V_{obs} of gelatin-coated quartz particles as a function of cell depth. Since, in this case, $V = U$, one should obtain a symmetrical parabola with value of zero at the positions of the cell walls. The data of Abramson *et al.* (32) are plotted in Fig. 5 for one such experiment, showing that $V = U$ under these conditions and confirming Smoluchowski's equation for liquid motion in a closed cell.

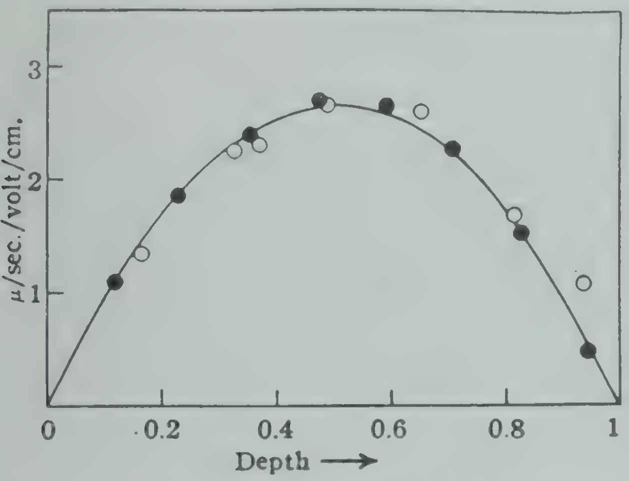

FIG. 5. The velocity of protein-coated particles in a flat electrophoresis cell coated with gelatin. The smooth curve is a parabola drawn on the theory that the velocity of electroosmosis is equal to that of electrophoresis. From Abramson *et al.* (32).

The minimum and maximum permissible thicknesses of a flat electrophoresis cell are determined by several considerations. The thinner the cell, the greater is the velocity gradient at the stationary levels and, therefore, the error in V_{obs} due to uncertainties in focus. However, the depth of the cell plus the thickness of the cell wall must be less than the working distance of the microscope objective. The above two requirements are compensatory, since the focusing error is less on smaller particles which require a higher magnification, which in turn means a shorter focal-length objective and, hence, a thinner cell. Cells of about 0.5 mm. thickness and 2 cm. width are suitable for particles ranging in size from the smallest visible to red blood cells (10 μ).

3. *Orientation of the Flat Cell*

For most purposes it is entirely satisfactory to place the flat electrophoresis cell in a horizontal position on the microscope stage. This orientation works perfectly well if the particles being observed are small enough so that they do not settle appreciably during their excursion across the field of vision of the microscope. However, if particles are of such size and density that they settle rapidly, then it is difficult to make accurate measurements. This led Abramson *et al.* (32) to propose that the electrophoresis cell be oriented with its long axis in a vertical direction. In such a case, settling and even convection tend to be parallel to the direction of observation and the particles do not enter layers with different liquid velocity attributable to electroosmosis, as explained above. With this arrangement, it is necessary to cancel out the effects of settling and of convection by making measurements in both upward and downward directions.

In 1952, Hartman and co-workers (33) pointed out that a third type of orientation of the cell was possible, namely, lateral orientation. In this case, both convective and settling disturbances are perpendicular to the direction of the velocity gradient of the liquid in the cell. By measuring excursion times between vertical lines in the microscope field, one can determine directly the electrophoretic component of the velocity of the particle and ignore the component in the perpendicular direction due to settling or convection. Although this orientation is superior in principle to the other two, in practice it has more convective disturbance than the horizontal for a given heat loss, probably because of the greater vertical distance in which convection can manifest itself. Also, convection currents can have lateral components. Whenever possible, the horizontal is the preferred orientation. The three types of orientation are illustrated schematically in Fig. 6.

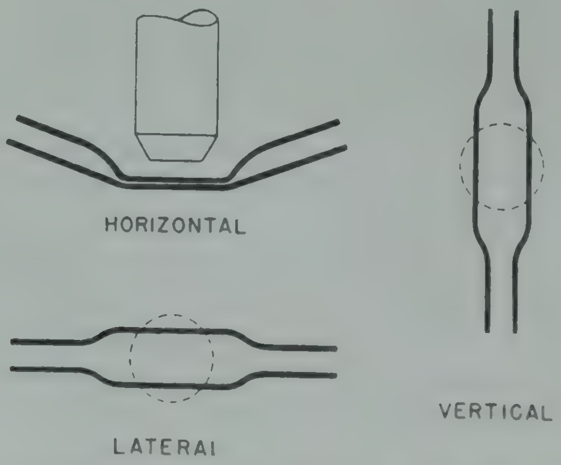

Fig. 6. Three orientations of the flat electrophoresis cell used in the microscope method. Plane of paper is vertical. From Hartman *et al.* (33).

4. Experimental Assemblies

The apparatus in most general use today is the Abramson (34) modification of the original Northrop-Kunitz wide, flat cell, a photograph of which is shown in Fig. 7. It is constructed entirely of Pyrex glass, which facilitates cleaning, and can be purchased from Klett Mfg. Co., New York City. The cell is made from 0.6-mm. Pyrex glass slides separated by 0.6-mm. Pyrex strips fused along the edges. The width : depth ratio is greater than 20:1. The whole assembly can be locked in a brass frame which rests directly on the microscope stage. Cu-$CuSO_4$ reversible electrodes are provided by a copper wire in contact with a copper sulfate solution, which is isolated from the cell by a plaster-of-Paris plug. Right-angle bore stopcocks at both ends provide a simple means of filling and cleaning without risk of contaminating the cell with electrolytes.

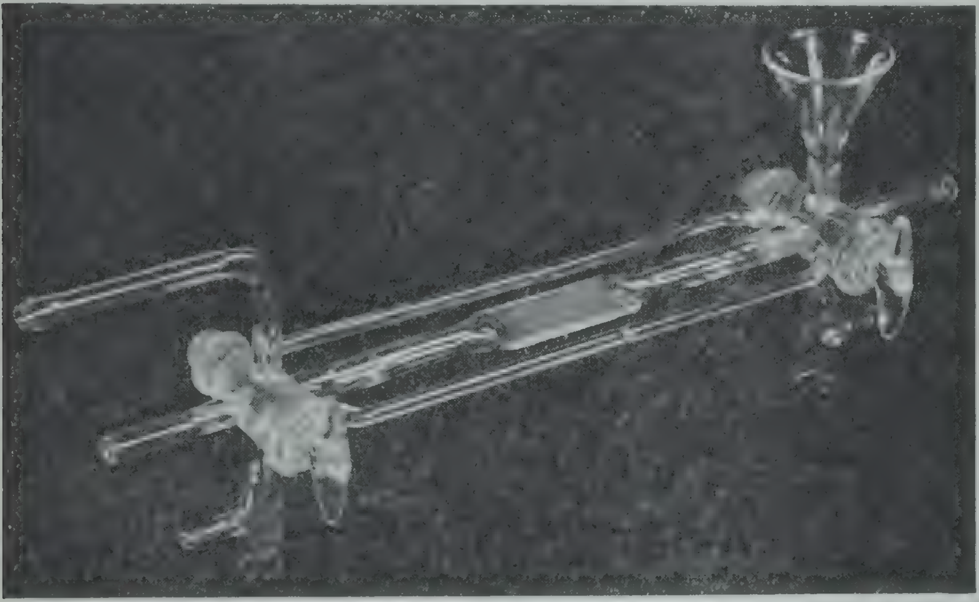

FIG. 7. The microscope electrophoresis cell of Abramson. Courtesy of Klett Mfg. Co.

A disadvantage of the Abramson apparatus is that it is adapted with difficulty to a vertical or lateral arrangement for the observation of rapidly settling particles such as blood cells. A simple experimental assembly for this has been described (33) which is the same as the Abramson apparatus except that it uses a simple Northrop-Kunitz cell attached to the rest of the apparatus by rubber tubing. If the cell is solidly attached to the microscope stage, vibration sometimes associated with rubber connections is eliminated. This apparatus is less readily broken because of greater flexibility and is easily repaired in case of breakage. The Northrop-Kunitz cell has been thoroughly tested by Abramson and Moyer and performs hydrodynamically according to theory. The mobilities obtained with it are in good agreement with values obtained independently by the moving boundary method (4, 35).

Another flat cell of considerable merit is the Chouceroun cell (36) illustrated in Fig. 8, which is made of a solid piece of glass 70 mm. × 45 mm. × 10 mm. into which two U-shaped channels are cut. Between the two inner arms of the U runs a small rectangular groove 0.07 mm. deep, 1 mm.

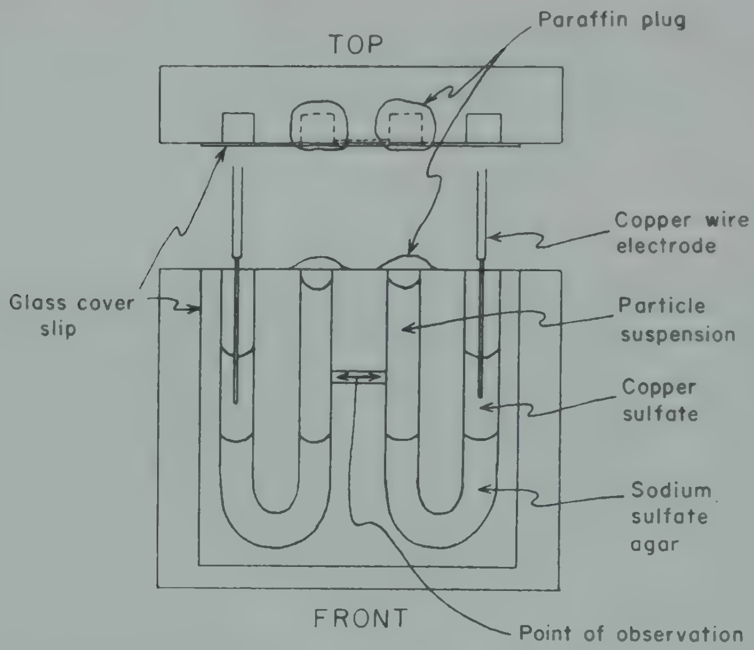

FIG. 8. The Choucroun microscope electrophoresis cell. The barrel of the microscope is oriented perpendicular to the plane of the paper above the observation channel shown on the front view.

wide, and 6 mm. long. The cell is manufactured in such a way that the groove is parallel to the face of the cell. It is assembled by adhering a cover slip, large enough to cover both U's, to the fact of the cell with molten paraffin, taking care that no paraffin runs into the groove. The bottoms of the U's are then filled with molten agar containing Na_2SO_4, which is then allowed to harden. The suspension to be examined is introduced above the hardened agar into the inner arms of the U's and the groove. This inner chamber is then sealed with a drop or two of very hot paraffin. $CuSO_4$ is introduced into the outer arms of the U's and copper wires are inserted as electrodes. During observation the whole apparatus rests on the microscope stage, the only connection being the two electrode wires. The Choucroun cell has the following advantages:

(1) Because it is thinner than the Northrop-Kunitz-Abramson cell, a higher magnification can be used, thereby increasing the sensitivity. Also, less current is required to obtain the same field strength, and measurements can therefore be made in higher salt concentrations without danger of convection.

(2) It is easily dismantled, cleaned, and reassembled. The electrode plugs are easily renewed.

(3) It is inexpensive and practically indestructible.

(4) It requires a very small amount of suspension (about 0.5 cc.)

(5) It is particularly well suited for pathogenic organisms since the complete apparatus can be autoclaved.

It has the disadvantage of having cell walls of different kinds of glass and more difficult focusing requirements. It is not suitable for large particles such as blood cells, but good results have been obtained in the authors' laboratory with bacteria.

The double-tube cylindrical cell of Smith and Lisse (37) as modified by Bradbury and Jordan (38), shown in Fig. 9, has been used by the latter authors to obtain reproducible mobilities. It employs, in parallel with the observation tube, an additional capillary tube whose length and diameter are such that no net flow of liquid occurs in the center of the observation tube. The theory of liquid flow in the Smith-Lisse cell is based on the same

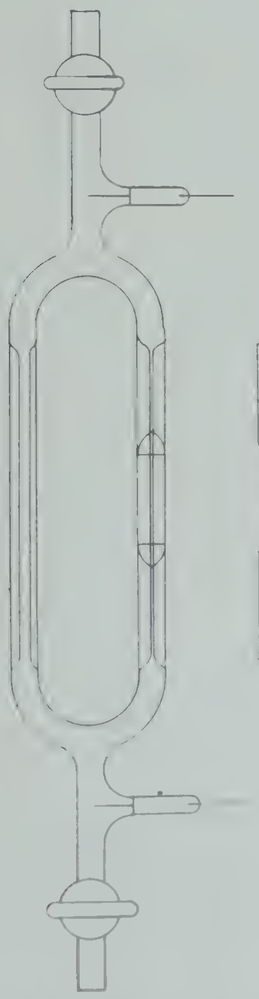

FIG. 9. The double-tube cell of Smith and Lisse. The cylindrical observation tube is ground flat on both sides at the point of observation. From Bradbury and Jordan (38).

principles used in deriving the equations for liquid flow in flat cells. The equation,

$$V_w = \frac{P}{4\eta l}(h^2 - a^2) + \frac{U'E'}{l} \tag{18}$$

is exactly analogous to equation (15) except that it applies to flow in a tube of circular cross section and that $U'E'/l$, where U' is electroosmotic mobility and E' is the potential across the tube, is substituted for its equivalent, U. When equation (18) is substituted into equation (15) and the integration performed, one obtains

$$\pi a^2 \left(\frac{U'E'}{l} - \frac{Pa^2}{8\eta l} \right) = 0 \tag{19}$$

If we have a cell consisting of two parallel tubes of radii, a_1 and a_2, and lengths, l_1 and l_2, respectively, which connect into the same large chamber at each end, the pressure, P, and the potential E' across both tubes will be the same and total volume of flow through both of them will be given by

$$\pi a_1{}^2 \left(\frac{U'E'}{l_1} - \frac{Pa_1{}^2}{8\eta l_1} \right) + \pi a_2{}^2 \left(\frac{U'E'}{l_2} - \frac{Pa_2{}^2}{8\eta l_2} \right) = 0 \tag{20}$$

The radii and lengths of the two tubes can be chosen such that V_w in the smaller tube (radius a_1) has a value of zero at the center, that is, where $h = 0$. When these conditions are substituted into equation (18), one obtains

$$U'E' = \frac{Pa_1{}^2}{4\eta} \tag{21}$$

When equation (21) is substituted into equation (20), one obtains upon simplification

$$l_2 = \frac{l_1(a_2{}^4 - 2a_1{}^2a_2{}^2)}{a_1{}^4} \tag{22}$$

If the ratio of the radii, a_2/a_1, is denoted by A, equation (22) becomes

$$\frac{l_2}{l_1} = A^2(A^2 - 2)$$

For tubes of equal length, $A = 1.55$.

In addition to the stationary level being in the center of the smaller tube, the velocity gradient there is zero, as can be seen by differentiating equation (18) and setting $h = 0$. The measurement of velocity at this level has the following advantages:

(1) The stationary level is readily found, since it is the level of maximum velocity. Focusing errors caused by the passage of light through a curved surface are, therefore, of no importance.

(2) Slight errors in focusing do not result in appreciable errors in velocities, since change of velocity with depth is small.

(3) Large particles do not move in liquid levels of different velocities since the gradient is small.

It is essential that both tubes be made of the same material, so that U' will be the same for each tube. Smith and Lisse used quartz because its index of refraction is nearer to that of water than glass, resulting in less optical distortion. The cell is easy to construct, clean, and fill. In spite of its advantages, however, it has not been widely used. The main disadvantage of the Smith and Lisse double-tube cell, that of optical distortion of images (39), can be overcome by combining a flat observation cell with a parallel flat or cylindrical compensating tube. Two other cells have been described in which the stationary level is at the position of zero velocity gradient: the double-path rectangular cell of Beniams and Gustavson (40) and the external capillary cell of Rutgers *et al.* (41). A new arrangement for microscope electrophoresis was described by Bangham *et al.* (41a) in which the cell is fixed permanently in position and is washed out *in situ.* A dial test indicator allows rapid focusing, and both the objective and the cell are immersed in water.

5. *Current Source and Determination of the Field Strength*

The electrical requirements for microscope electrophoresis are simple. All that is needed is a source of direct current smoothly variable from 0 to about 300 volts, a switch to reverse the current, and an accurate ammeter with several scales, preferably 0–10 microamperes, 0–100 microamperes, 0–1 milliamperes, and 0–10 milliamperes. Commercially available vacuum-tube ammeters best fulfill these requirements. Radio 45-volt B batteries are a satisfactory current source, although an electronically regulated direct-current power supply can be used. A satisfactory circuit has been described by Moyer (42).

The field strength is determined according to Ohm's law by measuring the specific resistance of the solution, the cross-sectional area of the cell, and the current. Specific resistance is measured in a platinum electrode conductivity cell by means of an alternating-current resistance bridge. The specific resistance is equal to R/K, where R is the resistance measured across the cell and K is a cell constant determined with a standard conductivity solution. The cell cross section at the point of observation is determined optically with a stage micrometer and the fine-focusing adjustment of the microscope.[3] Ohm's law in terms of the experimentally determined variables is, therefore:

$$E = iR/KA$$

---

[3] This measurement must be made on an air-filled cell.

where E = field strength (volts per centimeter), i = current in amperes, R = resistance across conductivity cell in ohms, K = conductivity cell constant, and A = cross-sectional area of electrophoresis cell.

6. *Microscope Optics and Focusing*

Almost any kind of optical arrangement is feasible when using flat, thin cells, subject, of course, to the restriction that the working distance of the objective must be at least as great as the depth of the cell plus the thickness of its roof. Magnifications of about 400–500 are commonly used, but 1000–1200 give better sensitivity and more accurate focusing. Higher magnification is usually obtained with a 40 X oil- or water-immersion objective and a 30 X ocular. Ordinary optics can be used, but phase contrast is much more satisfactory. Dark-field illumination or a slit ultramicroscope can be used for ultramicroscopic particles, such as viruses.

The distance the particle moves is measured with an ocular micrometer which is calibrated with a standard stage micrometer for the particular optical system in use.

One of the important sources of error is the determination of the stationary levels. Most ordinary microscope fine-focusing adjustments are linear over only part of their range, because of original construction and wear, and should, therefore, be calibrated with a standard-thickness cover slip. This can be done by measuring the thickness of the cover slip using different portions of the fine-adjustment range.

The portion of the range which measures true thickness should be used for obtaining the cell cross section. Since the stationary levels are relative to the cell depth, any linear portion of the range can be used for finding the stationary levels.

For a single observation, it is sufficient to know from previous measurements the cell depth at the point of observation and to find the stationary levels by measuring down from the ceiling and up from the floor, always using the same part of the range. The micrometer adjustment should be tightened sufficiently to prevent mechanical drift during observation.

7. *Sources and Magnitudes of Errors*

Lerche (8) analyzed extensively the errors involved in microscope electrophoresis. He found that the over-all reproducibility of duplicate mobility measurements on the same bacterial suspension on the same day was 2% and on different days was 5%. These values are in agreement with the authors' experience when particles of a homogeneous nature are examined; however, the error can be greater if a heterogeneous population is examined and the mobilities averaged. In the latter instance, it is better to express the results as a frequency histogram of individual mobilities. For a sufficiently homogeneous population, the average mobility for a single determination calculated using the average of transit times does not differ significantly from the average of the individual mobilities, if transit times in both directions and at both stationary levels are not much different. If these times are significantly different, as is sometimes the case, it is only correct to average individual mobilities, not individual transit times.

The chief sources of error in mobility measurements of homogeneous populations are: (1) inaccurate determination of stationary layers; (2) leakage, causing pressure differences; (3) heat generation, causing convection; (4) impurities in the central chamber.

Procedures for the elimination of most of these errors have already been discussed. Impurities in the central chamber are eliminated by thorough cleansing and rinsing of the cell and by proper isolation of the electrodes. The pH and conductivity of the particle suspension should be measured *after* the suspension has been withdrawn from the cell, as a control on impurities in the central chamber and buffer variations.

D. Mobilities of Submicroscopic Substances Adsorbed on Microscopic Particles

Many submicroscopic substances can adsorb onto the surface of microscopically visible particles. Examples are pure proteins (4), lipoproteins (43), antibodies (44), detergents (45), and viruses (46). Carrier particles include such diverse substances as quartz, glass, collodion, carbon, mineral oil, ferric oxide, ion exchange resins, silica gel, aluminum oxide, and even air bubbles. The mobility of adsorbed particles has considerable practical and theoretical importance. At the practical level, the microscope method, with its concomitant advantages over the moving boundary method, can be used to measure the mobility of submicroscopic substances; pH-mobility curves of proteins are commonly determined in this way. At the theoretical level, it gives some insight into the theory of charge density and ζ-potential calculations for large biological particles (Section II, D).

Carrier particles are coated by allowing them to remain in contact with a solution containing an excess of the adsorbate dissolved in a buffer solution. The extent of adsorption is a function of adsorbate concentration, and a saturation value of the adsorbate concentration is reached beyond which the mobility no longer increases. The mobility of a particle covered with adsorbate is characteristic of the adsorbed material and not of the particle. Carbon, quartz, mineral oil, glass, and collodion all have the same mobilities when coated with ovalbumin, while coated particles of silica gel and aluminum oxide show small deviations (47). As is usual for microscopic particles, coated particles migrate independently of size and gross shape.

The mobilities of dissolved proteins as measured by the moving boundary method are in many instances identical with mobilities of the same protein adsorbed onto a carrier surface. The pH-mobility curves of

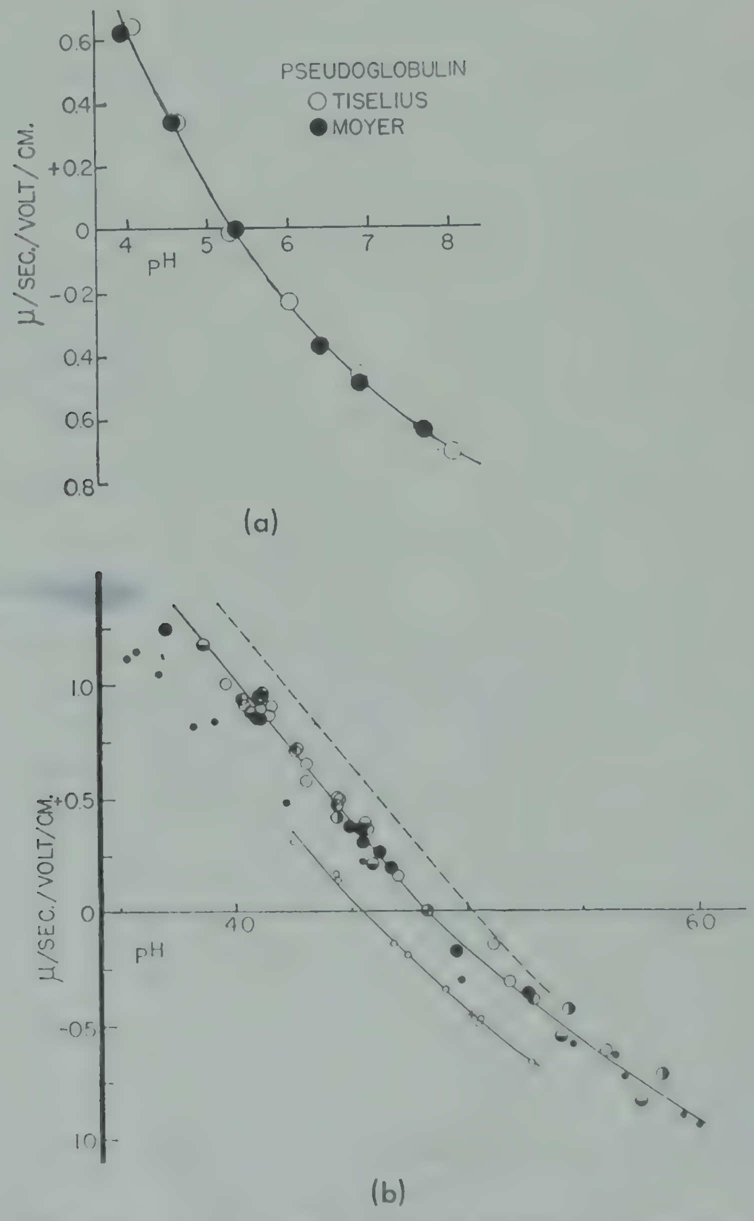

FIG. 10. Mobility-pH curves of dissolved and adsorbed protein. a. Horse pseudoglobulin; dissolved and adsorbed protein give exactly the same curve. b. Ovalbumin; the curve for the adsorbed protein is displaced to the right by 0.3 pH units but has the same slope. From Abramson *et al.* (4).

horse serum albumin and horse serum globulin are identical in the dissolved and adsorbed state (Fig. 10a). On the other hand, the pH-mobility curve of dissolved egg albumin is parallel to the curve for coated particles but shifted toward lower pH values by 0.3 units (Fig. 10b). This discrepancy has been attributed to denaturation of egg albumin at surfaces or to preferential orientation upon adsorption resulting in change of availability of charged groups (4). It is clear from these and other studies that mobilities and isoelectric points can sometimes differ from the dissolved to the adsorbed state, but that the pH-mobility curve retains its characteristic shape.

Bull (47a) has re-examined the relationship between the mobilities of adsorbed and dissolved proteins. He concluded that the mobilities of bovine serum albumin and of ovalbumin adsorbed on powdered pyrex glass varied with ionic strength in a manner requiring a radius of curvature much greater than the radii of protein molecules in solution. He concluded that Abramson *et al.* (4) overemphasized the agreement between the mobilities of dissolved and adsorbed protein but agreed, nevertheless, that these mobilities do not differ by the factor expected on the assumption that the radius of curvature is that characteristic of the adsorbent and the charge density that of the adsorbate. Bull's work demonstrates that the relationship between the mobilities of adsorbed and dissolved proteins is not yet completely clarified.

A possible application of the adsorption method is the detection of charge asymmetries. If, for example, two ends of a molecule carry different charges and also possess different affinities for carrier particles, depending on the carrier substance, one might expect the mobilities to vary in a characteristic way for different carrier particles. Coahran (43) adsorbed serum lipoprotein onto collodion, olive oil, a positively charged hydrophilic resin, and a negatively charged hydrophilic resin. If the lipid and protein portions of the molecule were asymmetrically disposed, a different mobility might have been found on hydrophobic and hydrophilic carriers. However, mobilities were the same for all four carrier particles, indicating no gross asymmetries.

IV. INFORMATION ABOUT VIRUSES, BACTERIA, AND CELLS OBTAINABLE BY ELECTROPHORESIS

A. Particle Interaction and Suspension Stability

The agglutination or precipitation of homogeneous suspensions of biological particles is conditioned by forces between the particles and between the particles and the solvent, which is assumed to be water. For purposes of discussion, it is usual to treat hydrophobic and hydrophilic surfaces separately. The stability of hydrophobic surfaces is considered to depend upon repulsive interactions between their electrical double layers and upon the attraction of van der Waals' forces. Since the van der Waals' forces decrease rapidly with distance, combinations of the two interactions give potential energy-distance curves with maxima at a given distance. The position and magnitude of the potential energy barrier are related to the nature of the surface and the magnitude of the ζ potential. Verwey and Overbeek (48), among others, have given a quantitative treatment of this problem. The particles will be stable when the potential energy barrier is greater than kT, but unstable when the maximum energy of the barrier is of the same order or less than kT. The lower the ζ potential, the greater will be the instability of the particles. Instability should be greatest at the isoelectric point.

Interaction with the solvent can be an important factor. A particle suspension might be stable even at its isoelectric point because the attractive forces between the particle and water are greater than the attractive forces between particles. Treatments which tend to reduce the attraction between the particle and the medium more than they reduce the attraction between particles will cause instability. Conversely, treatments which reduce the repulsion between the particles and the medium *less* than they reduce the repulsion between particles also favor instability. An example is the precipitation of protein by alcohol or concentrated ammonium sulfate. Particles are more easily precipitated by these agents if they are near their isoelectric point.

The relationship of ζ potential to stability is clearly seen with viruses. Although hydration can exert a stabilizing influence, the point of maximum insolubility nearly always coincides with the isoelectric point.

Striking confirmation of this was found by Oster (46), who measured the isoelectric points of various strains of tobacco mosaic virus by a turbidimetric method. Oster measured the relative turbidity of 0.2% virus solutions in $0.02\ M$ buffers as a function of pH. The turbidity-pH curves for the ordinary strain of tobacco mosaic virus (TMV) and for the PRS strain of influenza virus are shown in Fig. 11. The isoelectric point of TMV ordinary strain as given by the point of maximum turbidity is 3.5 ± 0.1. The turbidity increases because of the increased light scattering of the agglutinated virus particles. The isoelectric point of influenza virus measured by the above technique was pH 5.0 ± 0.1, and that of the Holmes rib-grass strain of tobacco mosaic virus was pH 4.0 ± 0.1. Oster states that "the isoelectric points of these viruses at the same buffer concentration were also determined by microelectrophoresis with the Kunitz-Northrop apparatus. In all these cases, the viruses, when adsorbed on collodion particles, give isoelectric points in agreement with those found

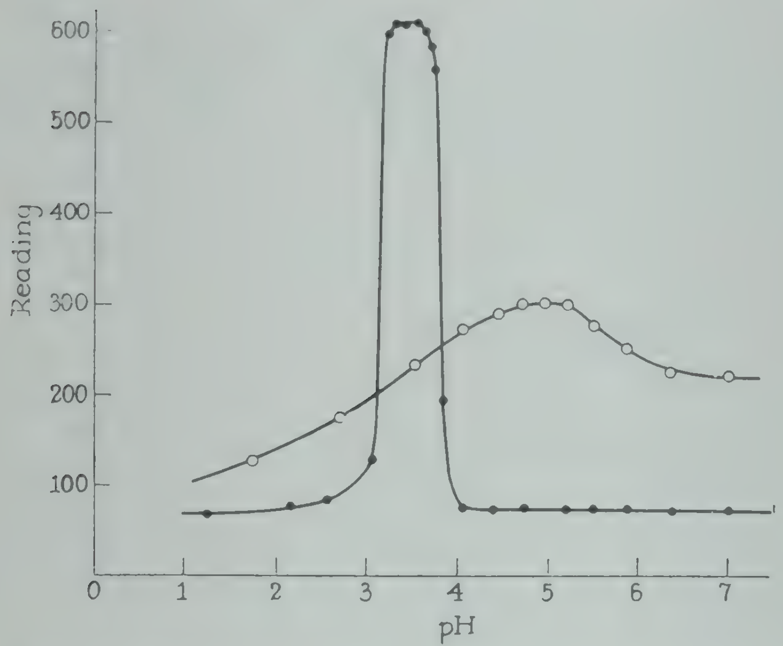

FIG. 11. Relative turbidities as a function of pH of solutions of the ordinary strain of tobacco mosaic virus (●) and of the PR8 strain of influenza virus (○). From Oster (46).

by the turbidimetric method." Though the pH of the isoelectric point can be somewhat different in different buffers, even at the same ionic strength, the pH of maximum insolubility is still the pH at which no movement occurs in electrophoresis. The spherical plant viruses—tomato bushy stunt, tobacco necrosis, southern bean mosaic, and turnip yellow mosaic—are all soluble at their isoelectric points, presumably because of hydration.

B. Determination of the Nature of the Unaltered Particle Surface by Measuring the Mobility as a Function of pH and Salt Concentration

The electrokinetic quantity which reflects most accurately the nature of the particle surface is the average net surface charge density, σ. σ-pH curves and σ-salt concentration (C) curves might, therefore, be expected to yield valuable information. However, there are several characteristics of the surface charge density of biological particles which greatly reduce the amount of information to be obtained from σ-pH or σ-C curves.

(1) Non-specific charging mechanisms can make large contributions to σ. (For instance, "neutral" surfaces such as gas bubbles and paraffin particles can have comparatively large, pH-dependent charges.)

(2) Biological surfaces are usually complexes of different kinds of substances, and σ reflects only the average surface composition.

(3) σ is difficult to determine with any degree of accuracy because of

its strong dependence on particle dimensions and because of our lack of knowledge about the surface dimensions of biological particles.

A combination of biochemical and electron microscopical analysis can give much more reliable information about the nature of particle surfaces than can σ-pH or σ-C curves.

C. Alteration of Particle Surface as Followed by the Change in Mobility

1. Alteration by Biological Treatment

Viruses of the mumps-influenza group have the ability to cause the agglutination of red blood cells. After the initial agglutination, the virus is spontaneously eluted, leaving the cells in a modified state in which they can no longer be agglutinated and in which they can adsorb more virus. It was discovered by Hanig (49) that cells treated with virus have a reduced electrophoretic mobility. The electrophoretic mobility of the cells and the virus titer of the supernatant solution after the cells have been removed by centrifugation were followed as functions of time. The results are shown in Fig. 12. The rate of mobility decrease and the final mobility value are both dependent upon virus concentration. The decrease in mobility has been shown to be a direct result of the destruction of the virus receptor on the red cell surface. Bateman *et al.* (50) have attributed the decrease in net negative charge density to the formation of additional free positively charged groups.

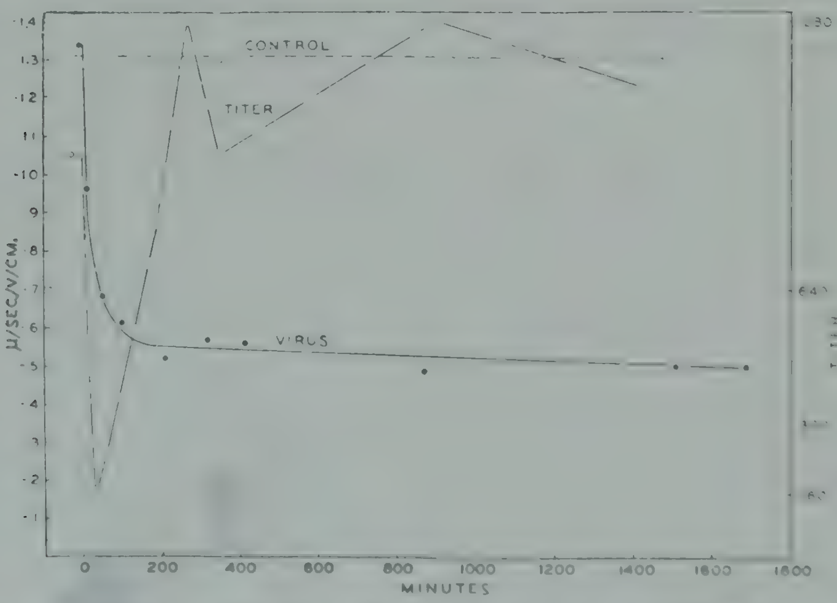

Fig. 12. Decrease in human red blood cell mobility in the presence of PRS influenza virus. The mobility (●) is plotted as a function of time of contact. From Hanig (49).

2. Alteration by Chemical Treatment

a. Adsorption of a charged substance. The possibilities of electrophoretically identifying the different surface components of biological particles are greatly increased by the use of a technique employing molecules of known charge with a specific affinity for certain substances. The particles to be investigated are suspended in solutions containing various concentrations of these molecules and their mobility is measured. If the substance for which the molecules are specific is present on the particle surface, the molecules are adsorbed and the mobility is changed in proportion to the amount of substance present. An excellent example of this technique can be found in the work of Dyar and Ordal (51) and Dyar (45). As charged indicators, the anionic detergents, dodecyl, tetradecyl, and hexadecyl sulfonic acids, were employed by these workers. Their theory was that the hydrocarbon end of these molecules would be specifically adsorbed to surface lipid and that the negatively charged end would increase the net negative charge on the particle, which in most cases was the bacterium, *Micrococcus aureus*. When the anionic detergents were present in the suspension, the mobility of the bacteria indeed increased. Droplets of pure hydrocarbons and lipids also increased their negative mobility in the presence of the detergents. In Fig. 13 the increase in mobility with con-

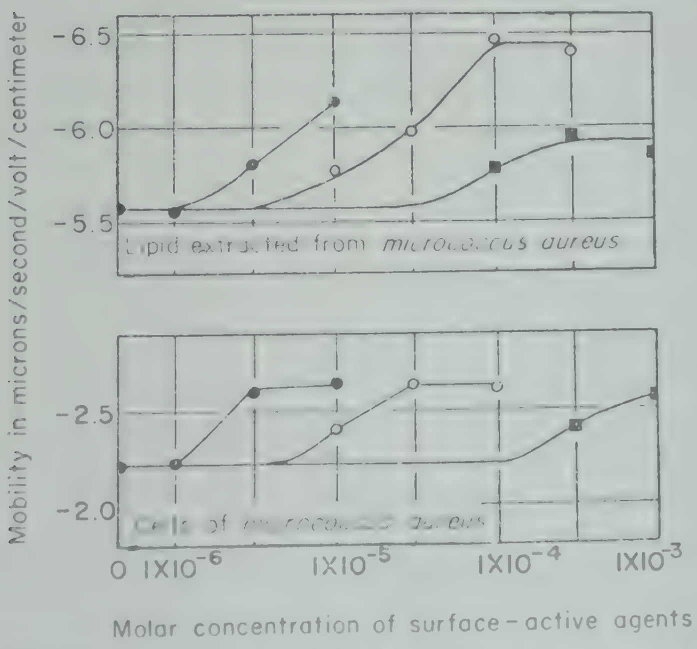

FIG. 13. Mobility-concentration curves of *Micrococcus aureus* and of lipid extracted from the same organism, treated with sodium hexadecyl sulfonate (●), sodium tetradecyl sulfonate (○), and sodium dodecyl sulfonate (■). From Dyar (45).

centration of detergent is shown for *Micrococcus aureus* cells and for pure lipid extracted from the same cells. Although the initial charge is not the same because of the presence of other materials which influence it, the cell's electrophoretic response is very similar to that of the lipid. To determine the specificity of this method, proteins (egg albumin and bacterial nucleoprotein), polysaccharides (cellulose and dextran of bacterial origin), and an "inert" surface (Pyrex glass) were tested. The polysaccharides and the Pyrex particles showed no increase in mobility in detergent solutions at any pH. The charge on the protein did not increase in the presence of detergents as long as the pH was sufficiently high for the maximum number of negative charges to exist, but at more acid pH values the charge increased, presumably due to neutralization of some of the positive groups of the protein by the negatively charged detergent. This technique offers the possibility of detecting lipid surface components as long as the pH is kept high enough to prevent the ionization of the positively charged groups of amphoteric substances (protein in biological particles). There is no reason in principle why a similar technique could not be developed to detect other components.

b. Specific removal of a given component. There are treatments (chemicals, enzymes, shaking) which will specifically remove a certain surface component. If this component contributes to the mobility, the mobility should change in a characteristic way upon its removal. From knowledge of the specificity of the agent, the component can be identified.

i. Chemical. The enzymes, lipase and trypsin, have been used by Dyar (45) to effect the specific removal of lipid and proteinaceous material, respectively, from the *Micrococcus aureus* surface. After treatment with lipase, the negative mobility of the bacteria no longer increased when anionic detergents were present, indicating removal of lipid. The dependence of bacterial mobility on pH was greatly reduced by treatment with trypsin, indicating the removal of amphoteric material.

The usefulness of the specific removal method depends upon the effectiveness and specificity of the agent used to remove a particular component. Trypsin, for instance, did not remove amphoteric material from *Mycobacterium smegmatis*, and petroleum ether extraction had little effect on the lipid of *Micrococcus aureus*.

ii. Mechanical. The mechanical removal of a specific surface component has been used to demonstrate the effect of this component on the mobility of the particle. Brinton *et al.* (24) found from electron microscope investigations that the capsular hairlike filaments of *Escherichia coli* (Fig. 2) could be almost completely removed by shaking in a high-speed mixer. These defilamented bacteria had a mobility identical with a nonfilamented variant of the bacterium whose mobility was higher than the filamented form, showing that filaments decreased the mobility of the bacteria on which they grew. After removal by shaking, the filaments could regrow, the extent of regrowth determining the mobility of the organism.

D. Adsorption of Exogenous Substances on Particles as Determined by Mobility Measurements

The measurement of change in particle mobility due to an adsorbed substance, as discussed in Section III, has a valuable application in the titration of antisera against the particles. Coulter (52), de Kruif and Northrop (53), and Shibley (54) were able to demonstrate that homologous antiserum reduced the mobility of bacteria while heterologous antiserum did not. Recently, Hartman and Nungester (44) have adapted this principle for the assay of antisera against cells of lymphosarcoma ascites tumor. In this method, various dilutions of antiserum are added to a cell suspension in a suitable buffer. At high dilutions, the cell mobility will be the same as in buffer alone; at low dilutions, the surface will be saturated with antibody and mobility will be reduced to a minimum, because adsorbed γ-globulin has a considerably lower charge density than the cell surface. At intermediate dilutions, the mobility will depend upon antiserum concentration. The serum concentration: mobility ratio curve of Hartman (55) is shown in Fig. 14. An arbitrary value of the mobility (on a steep portion of the curve for greatest accuracy) can be chosen as

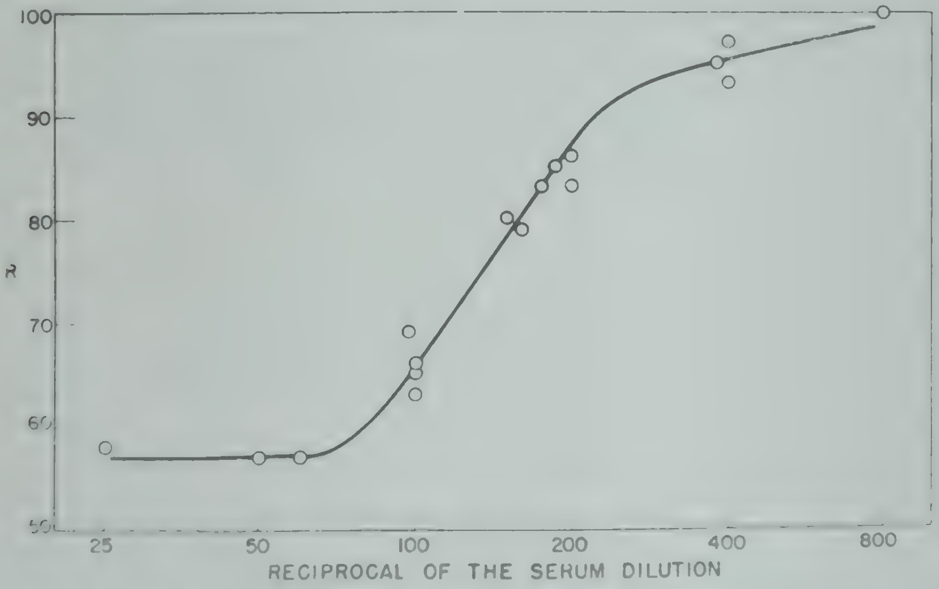

FIG. 14. The effect of homologous antiserum upon the mobility of Gardner lymphosarcoma cells. The ordinate, *R*, is the ratio of the mobility in a given dilution of antiserum to that in buffer alone, the former mobility having been corrected to the ionic strength of the buffer. From Hartman (55).

the end point of the serum titration. A comparison of the electrophoretic end point with a neutralization end point showed proportionality between the two within the limits of experimental error, the electrophoretic test being about five times as sensitive as the neutralization test.

The types of information discussed thus far in this section are concerned with the nature of the particle surface itself or of material adsorbed upon it. The following types of investigations depend only upon the separation of particles with different mobilities.

E. Following the Course of a Reaction by Electrophoretically Separating the Reactants, Intermediates, and Products

Electrophoresis is a very powerful tool in the study of kinetics, because various components in reactions involving biological material can be followed as a function of time. The properties of electrophoresis which are desirable in this respect are:

(1) The concentrations of the various components can be measured in the Tiselius apparatus.

(2) The passage of an electric current large enough to separate components does not affect their activity or structure.

(3) Electrophoresis is capable of detecting relatively small charge differences.

The usual procedure is to separate the components electrophoretically at various times after the reaction has started. Intermediates and products must be identified by independent methods after they are mechanically isolated from the separated mixture. The analysis of the alkali splitting of tobacco mosaic virus by Schramm *et al.* (56) is an example of this method.

A purified suspension of tobacco mosaic virus is held in pH 10.3, 0.05 *M* carbonate buffer for various lengths of time and then subjected to electrophoresis in the Tiselius apparatus. The schlieren diagrams in Fig. 15 show the number of components and their change in concentration with time. Schramm has isolated the five components and identified them in order of decreasing mobility as pure ribonucleic acid, two alkali-labile virus intermediates, an alkali-stable high molecular weight nucleoprotein, and pure low molecular weight "A" protein. As the decomposition proceeds, the alkali-labile virus fractions disappear, the concentration of "A" protein and pure nucleic acid increases, and the alkali-stable nucleoprotein fraction remains the same. This operation permits the simultaneous purification and separation of tobacco mosaic virus protein from the viral nucleic acid.

Electrophoresis in the Tiselius apparatus has been employed by several authors to follow the intracellular growth of virus (57-63). The electro-

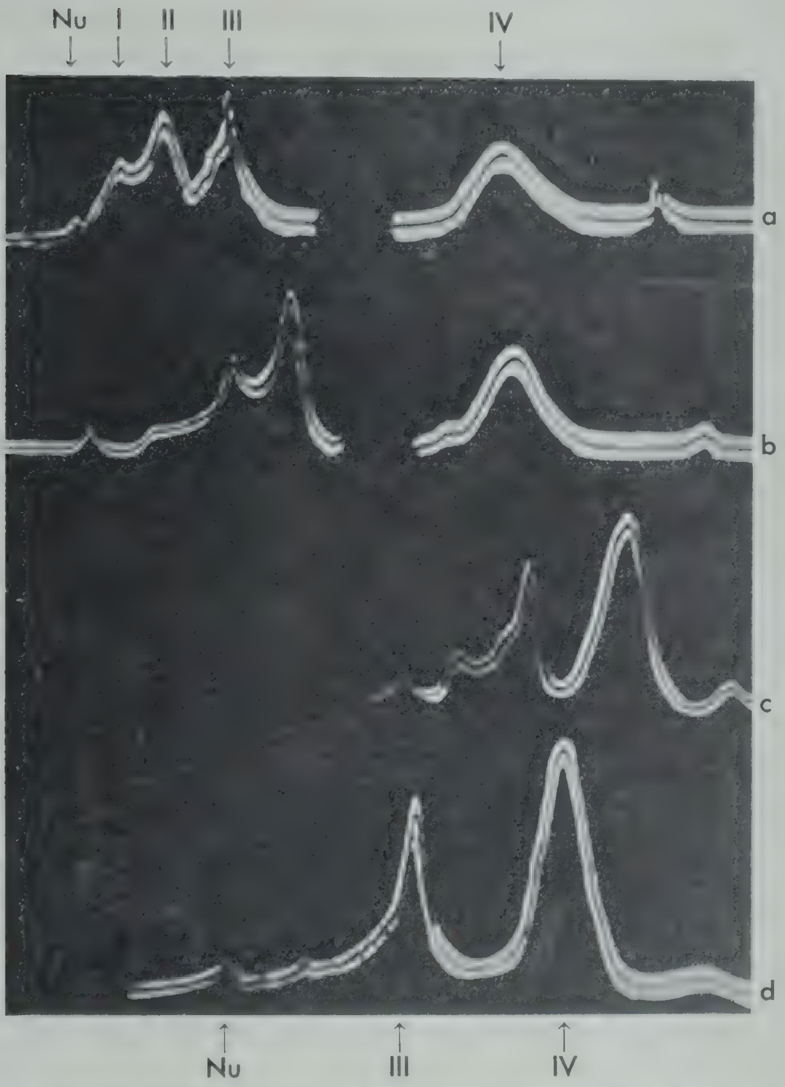

FIG. 15. Electrophoresis diagrams of tobacco mosaic virus after various times in contact with 0.05 *M* carbonate buffer at pH 10.3. Cathodic limb of the U-tube, direction of migration from right to left. *a*. 1 hour. *b*. 8 hours. *c*. 16 hours. *d*. 72 hours. Nu = free nucleic acid; I and II = alkali-labile virus fractions; III = alkali-stable virus fraction; IV = nucleic acid-free, low molecular weight protein. From Schramm *et al.* (56).

phoretic analyses by Wildman and Bonner (64) of leaf cytoplasmic proteins as a function of time after virus infection may be taken as an example. The schlieren diagrams of these preparations are shown in Fig. 16. The more diffuse peak is normal tobacco plant ribonucleoprotein, and the sharp peak is tobacco mosaic virus. The decrease in normal protein and increase in virus protein is taken to mean that the virus protein is formed at the expense of the normal protein. The initial appearance of the virus peak at 3 days is well before the appearance of external signs of disease, making electrophoretic analysis potentially valuable as a diagnostic test.

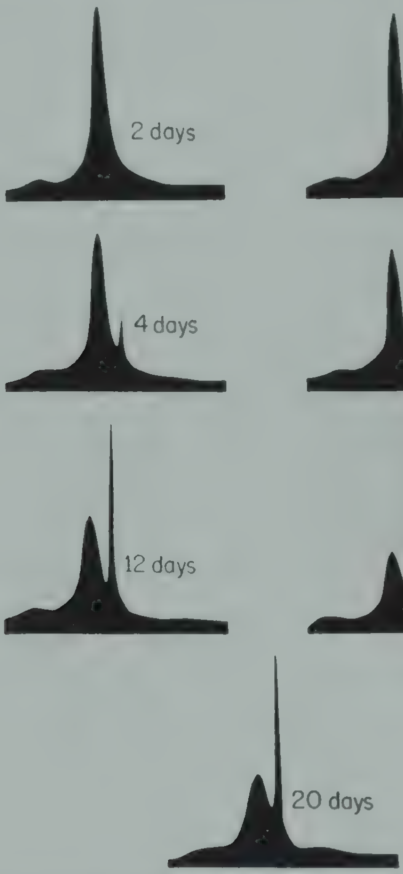

FIG. 16. Electrophoretic scanning patterns of whole tobacco leaf cytoplasm prepared at various intervals after infection with tobacco mosaic virus. From Wildman and Bonner (64).

F. Inhomogeneities in Particle Populations

Electrophoresis is a very sensitive technique for detecting differences in the surfaces of viruses, bacteria, or cells brought about by biological variation or chemical treatment. Differences in the surface charge density of mutants or chemically treated particles can be resolved by either the microscope or moving boundary methods. If an electrophoretic inhomogeneity is observed, it is virtually certain that a difference in surface constitution or structure is involved. Clean-cut distinctions between classes of particles in a mixed population are frequently obtained.

An example of the resolution of mutant organisms can be found in the electrophoretic study of bacterial "dissociation." It has been found by many investigators (20, 25, 65, 66) that rough-colony-forming "R" variants of bacteria usually have a greater mobility than the corresponding smooth colony forming "S" variants. The formation of S colonies has been associated with the production of capsular material, and their smoother appearance is probably associated with an increased hydration in the colonial form. The production of a capsule that will move kinetically with the particle can have a large effect on the particle's mobility, because the capsular material is usually of quite different constitution than the cell wall. It has been shown that the capsular filaments of *Escherichia coli* cause the variant producing them to have smoother colonies and a lower electrophoretic mobility (20, 24) than the variant which does not produce them. The distribution of mobilities in a mixed population of R and S forms of *Escherichia coli* is shown in Fig. 17, illustrating the clear-cut separation. Electrophoresis can be used to count the relative numbers of each form in a population.

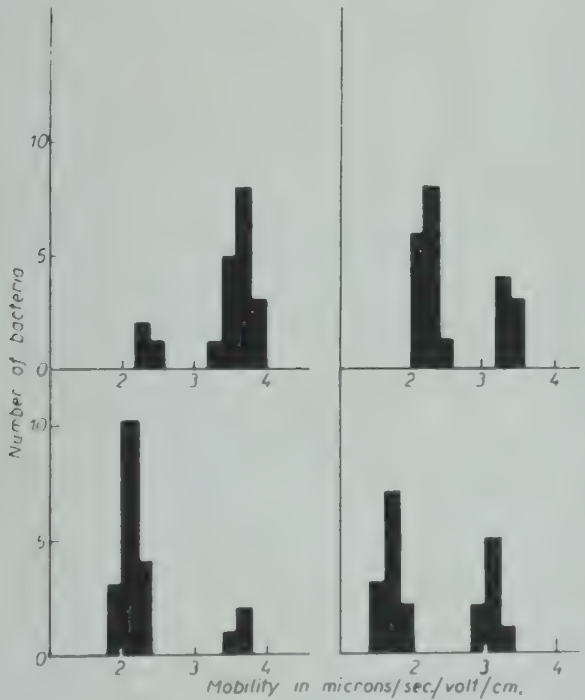

FIG. 17. Electrophoretic mobility distributions of heterogeneous populations of filamented and nonfilamented *Escherichia coli*. From Brinton *et al.* (24).

It must be emphasized that by no means all bacterial variations are detectable by electrophoresis—not even those affecting surface properties. For example, mutants of *Escherichia coli* resistant to the T-series of bacteriophages are electrophoretically indistinguishable from the parent strain (20, 67) although the surface structure is presumably different. However, if the theory of Puck (68) is correct, specific phage adsorption depends upon a strictly local pattern of positive and negative charges.

As electrophoresis measures only the average net surface charge density, such local differences cannot be detected, illustrating one of the fundamental limitations of the method.

The same situation obtains with the viruses; some mutants are electrophoretically different and some are not. The rib-grass strain of tobacco mosaic virus has a different isoelectric point from the type strain (46, 69), while a variant of southern bean mosaic virus which produced different symptoms from the parent strain had an identical pH-mobility curve (70).

G. Identification of a Property with a Characteristic Particle

An impure suspension of particles might have one or several biological properties associated with it; for example, infectivity and hemagglutination. It is often unknown whether or not a given property is associated with a given particle. Electrophoresis can be used to help establish this identity. If the mobility of the property is the same as the mobility of the particle, within experimental error, this constitutes evidence that the property belongs to the particle and not to some impurity. This type of evidence has been used to identify hemagglutinating activity with the influenza virus particle (71).

The technique of this method has been worked out in detail by Hartman and Lauffer (72), who determined the association of infectivity with the characteristic particle of southern bean mosaic virus. The migration rate of infectivity was determined by taking 0.1-ml. samples from the Tiselius cell with a long needle and testing them for infectivity. Samples were taken from positions just above and just below the position of the virus peak in the cell. The mean ratio of lesions produced by samples taken just below to samples taken just above the peak was 26, showing the coincidence of particle and infectivity mobilities.

V. RESULTS OF RESEARCH

A. Viruses

1. General

Plant and bacterial viruses consist of protein and nucleic acid, with the protein on the outside. Their electrophoretic behavior will, therefore, be that of the proteins, that is, amphoteric and hydrophilic. Some animal viruses also contain lipid but, in all probability, their external surface is mainly, if not entirely, protein. Because the isoelectric point is of practical value from the standpoint of solubility, we have compiled a table of virus isoelectric points (Table III).

Unlike bacteria and cells, which are nearly always observed by microscope electrophoresis, virus migration can be observed by any of the

TABLE III

Virus Isoelectric Points

Virus	Isoelectric pH	Ionic strength	Buffer	Reference
Tobacco mosaic (TMV)	3.49	0.29	Sodium acetate	(19)
TMV strain 2A	3.5	0.10	Sodium acetate	(73)
TMV ordinary strain	3.5	0.02 *M*	Citrate	(47)
TMV-Holmes rib grass	4.0	0.02 *M*	Citrate	(47)
TMV ordinary strain	3.91	Very low	Water + HCl	(47)
TMV-Holmes masked	3.91	Very low	Water + HCl	(47)
TMV-J14D1	4.22	Very low	Water + HCl	(47)
TMV-Holmes rib grass	4.49	Very low	Water + HCl	(47)
TMV-green aucuba	4.50	Very low	Water + HCl	(47)
TMV-yellow aucuba	4.64	Very low	Water + HCl	(47)
TMV-cucumber virus 4	4.90	Very low	Water + HCl	(47)
Southern bean mosaic (SBM) Princeton strain	5.50	0.02	Sodium acetate	(74)
SBM-Pittsburgh strain regular	5.9	0.02	Sodium acetate	(70)
SBM-Pittsburgh strain yellow	5.9	0.02	Sodium acetate	(70)
SBM-Pittsburgh strain regular	5.9	0.02	Sodium acetate and sodium phosphate	(72)
Tomato bushy stunt	4.11	0.02	Sodium acetate	(75)
Alfalfa mosaic	4.6	0.05 *M*	Phthalate-phosphate borate	(76)
Turnip yellow mosaic	3.7			(77)
Tobacco ringspot	4.7			(78)
Influenza A PR8	5.30	0.02 *M*	Acetate	(71)
Influenza A PR8	5.0	0.02 *M*	Citrate	(47)
Rabbit papilloma	5.0	0.10	Veronal acetate	(80)
T6 Phage	<4.6			(81)

electrophoretic methods. Early workers (73–75) followed virus migration in single cylindrical tubes by means of infectivity and serological tests. This method, with various refinements to allow for accurate sampling and prevention of convection, is still used (72, 76, 77). The mobility of viruses adsorbed on collodion particles has been measured by the microscopic method (46, 71, 78, 79) and, in every instance thus far tested, the mobility has been the same as that of the virus elementary particles. The larger viruses, such as vaccinia or the phages, T2, T4 and T6, can be visualized in the ultramicroscope by dark-field illumination, but to the authors' knowledge this very advantageous technique has never been used. Most virus electrophoresis has been done in the Tiselius apparatus using schlieren or absorption photography. Recently, zone electrophoresis of viruses on paper (80, 81), in sucrose density gradient columns (82), and in pH gradient columns (83) has been investigated.

2. *Plant Viruses*

a. Mobility measurements. Among the plant viruses, the virus of tobacco mosaic has received the most attention. Takahashi and Rawlins (84) used a crude type of apparatus, the Todd U-tube (73), to determine the electrophoretic behavior of TMV by infectivity measurements. With the advent of the Tiselius method of electrophoresis, which eliminated convective disturbances and allowed boundaries to be followed optically, exact quantitative mobility measurements became possible. Using the Tiselius apparatus and absorption photography, Eriksson-Quensel and Svedberg (18) determined a precise pH-mobility curve (Fig. 18) of a crystalline sample of TMV in the region of the isoelectric point.

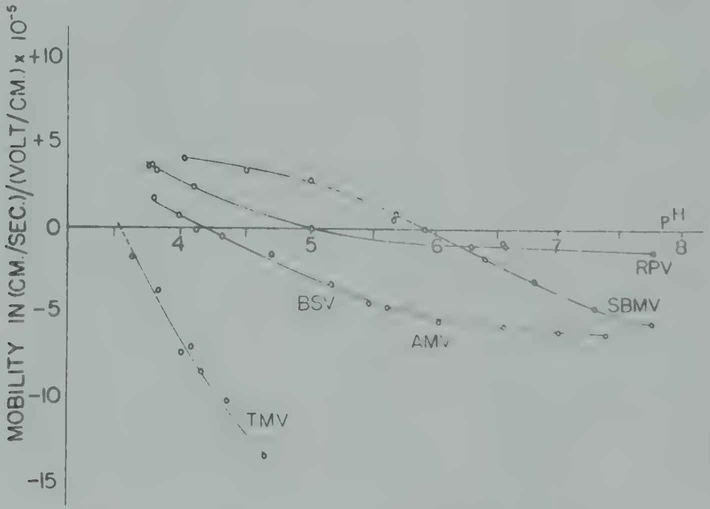

FIG. 18. pH-Mobility curves for viruses. TMV, tobacco mosaic virus; BSV, bushy stunt virus; AMV, alfalfa mosaic virus; SBMV, southern bean mosaic virus; RPV, rabbit papilloma virus. Salt concentrations are different for each curve.

McFarlane and Kekwick (85) determined the pH-mobility curve of a crystalline preparation of tomato bushy stunt virus (Fig. 18) using the Tiselius apparatus and simple schlieren photography. The virus was electrophoretically homogeneous in the pH range examined (pH 3.8–5.5).

Lauffer and Ross (86) measured the mobility of alfalfa mosaic virus in the Tiselius apparatus, using schlieren scanning photography (Fig. 18). The isoelectric point was determined by solubility measurements to be pH 4.6. It could not be found by moving boundary electrophoresis because of insolubility and instability below pH 5.5.

Miller and Price (87) determined the pH-mobility curve of southern bean mosaic virus (Princeton strain). Above pH 12.5 and below pH 2 the virus showed more than one component because of degradation, but within this range it was quite homogeneous.

b. Strain differences. Electrophoretic mobility has been found to be constant for a given strain of virus at a given pH and ionic strength but usually varies from strain to strain. Knight and Lauffer (69) showed that the ordinary and rib-grass strains of TMV had different mobilities under the same conditions: -8.9 and -7.8×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, respectively, in 0.05 *M* phosphate at pH 7.1. However, the proteins isolated from the two strains by alkali treatment had the same mobilities, even though their amino acid analyses were considerably different. Although the protein cleavage products were quite inhomogeneous with respect to size, they were unresolvable electrophoretically. Kahler and Woods (88) used the electrophoretic technique to separate the ordinary strain of TMV from a strain having the ability to form nuclear inclusions. MacDonald *et al.* (70) discovered that a spontaneous mutant of southern bean mosaic virus (Pittsburgh strain) had a different pH-mobility curve than the parent (Princeton) strain. However, a variant of the Pittsburgh strain producing yellow color in infected leaves had exactly the same pH-mobility curve. Miller *et al.* (89) confirmed the pH-mobility curve of MacDonald, Price, and Lauffer for the Pittsburgh strain of southern bean mosaic virus.

c. Zone electrophoresis. For the complete separation of a virus from another strain, host substances, degradation products, or impurities, zone electrophoresis has certain advantages over moving boundary electrophoresis as a preparative method. The use of a supporting medium to prevent convective disturbances and the longer distance through which migration can take place allows the components of a mixture to be completely separated even though their mobilities are not very different. Zaitlin (81) has taken advantage of the different mobilities of two strains of TMV (90) in order to separate them by paper electrophoresis. Gray (80) used one-dimensional paper electrophoresis to analyze the leaf cytoplasmic proteins in healthy and diseased plants. He was able to confirm the presence of new uninfectious proteins in infected leaves. The electrophoresis of potato yellow dwarf virus in sucrose density gradient columns has been investigated by Brakke (82). This method has the advantage that the virus migrates as a zone and can be removed quantitatively without elution.

d. Effect of chemical treatment. The effect of chemicals on the mobility of TMV has been determined for phenyl isocyanate, ketene, carboxypeptidase, and formaldehyde. In most cases, the mobility is altered enough to give separate peaks in the schlieren diagram for mixtures of treated and untreated virus, but the treated virus remains electrophoretically homogeneous. Miller and Stanley (91) obtained infectious acetyl and phenylureido derivatives of TMV by treating with ketene and phenyl isocyanate. Electrophoresis in the Tiselius apparatus in 0.1 ionic strength, pH 7.3 phosphate-KCl buffer showed the mobility of the unaltered virus to be -8.3×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, while the two derivatives each had a mobility close to -9.3×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. The derivative preparations were homogeneous, containing no appreciable amount of unchanged virus. Fischer and Lauffer (92) treated TMV with formaldehyde at pH 7.0 and pH 4.0. Electrophoresis was carried out in 0.2 ionic strength phosphate buffer at pH 7.00. Virus treated at pH 7.00 had an increased anodic mobility but was homogeneous. The anodic mobility increased as a function of time from -7.20×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ for the untreated virus to -7.58×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ after 24 hours' treatment at room temperature. Virus treated at pH 4.0 had an altered mobility and was inhomogeneous. Harris and Knight (93) found that by treating TMV with the enzyme carboxypeptidase 2900 ± 100 threonine residues were released per virus particle when the reaction was carried to completion. Threonine only was released by this treatment and the biological properties, size, shape, and density of the treated virus were identical with the untreated virus. The electrophoretic mobility and serological properties were altered, however. The mobilities as measured in 0.1 ionic strength, pH 6.9 cacodylate buffer were, for untreated virus, -7.4×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ and, for treated virus, -7.9×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. Mixtures of the two could be resolved.

It is of some interest to note that all four chemical treatments discussed above increased the anodic mobility of TMV by approximately the same amount (5–10%).

e. Analysis of in vivo virus growth. A great deal of attention in recent years has been focused upon the production in TMV infected leaves of certain noninfectious proteins which are not found in healthy leaves and are serologically related to TMV. The most fruitful method for demonstrating the presence and determining the relative amounts of these proteins has been electrophoretic separation in the Tiselius apparatus. Frampton and Takahashi (94) obtained schlieren scanning patterns of the cytoplasmic proteins of healthy and TMV infected tobacco leaves. They found three components (A, B, and C) in healthy leaves and four components (A, B, C, and V) in diseased leaves, component V being identifiable with the virus. Wildman and his co-workers (58), on the basis of a more quantitative extraction method for cytoplasmic proteins and a greater number of experiments, were able to show that the TMV component reached its maximum concentration 12–14 days after inoculation and increased in amount at the expense of cytoplasmic protein. These authors found only one component in healthy leaves and two in diseased leaves, one of which was the virus. Commoner *et al.* (61), Takahashi and Ishii (95), Commoner *et al.* (62), and Jeener *et al.* (63) have detected the presence in TMV-infected leaves of low molecular weight, noninfectious protein components serologically related to TMV but containing no nucleic acid. These components can be repolymerized at low pH values to form high molecular weight viruslike rods in the same manner as the protein obtained from TMV by alkali treatment. The electrophoretic mobilities of the protein components and of TMV are all different, allowing them to be quantitatively separated in the Tiselius apparatus. The low molecular weight component B3 of Commoner *et al.* (62), which is identical with the "X" protein of Takahashi and Ishii, has a mobility in 0.05 M, pH 7.0 phosphate buffer of -3.4×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. In the repolymerized state the anodic mobility increases to -8.0×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, indicating either a change in surface charge due to repolymerization, or a change in hydration (96). A comparison among the above authors of the relative amounts of protein and virus components occurring as a function of time after infection shows discordant results, probably due to differences in isolation procedure and physiological condition of the leaves. Since repolymerization changes the mobility, the same protein might appear as two components depending on its state of aggregation.

3. Animal Viruses

a. Influenza virus. By means of mobility measurements at a limited number of pH values, Bourdillon (76, 97) found that the mobility of PR8 influenza virus and its complement-fixing antigen were the same. He used the infectivity sampling method in the Tiselius apparatus. Miller *et al.* (71) made a thorough study of PR8 influenza virus both by the moving boundary and the microscope methods. The moving boundary method revealed that crude influenza virus preparations contained two electrophoretically distinct components: one of which was the pure virus present to the extent of 80-90%, and the other was an impurity similar to or identical with a high molecular weight impurity present in uninfected material. The two components could be separated either by ultracentrifugation or electrophoresis into fractions which were homogeneous in the ultracentrifuge and the Tiselius apparatus. The mobility of purified virus was the same whether observed as a moving boundary or adsorbed onto collodion particles, except for a slight divergence at high pH values. The isoelectric point by either method was pH 5.3. Wehmeyer (98) has reported that a component present in influenza virus-containing solutions similar to the high molecular weight impurity of Miller and co-workers, dissociates into two independently migrating components under the influence of the electric field.

b. Poliomyelitis virus. Schwerdt (99) used moving boundary electrophoresis as one step in a purification procedure for the production of crystallizable poliomyelitis virus from tissue culture fluid. Electrophoresis of a partially purified preparation of the MEF-1 strain consistently showed four components, infectivity being associated with the component of lowest mobility. Polson (100) has succeeded in concentrating and partially purifying MEF-1 poliomyelitis virus in a continuous flow electrodecantation apparatus.

c. Vaccinia virus. Smadel *et al.* (78, 101) have shown that the elementary bodies of vaccinia migrate as a single discrete boundary in the Tiselius apparatus. Collodion particles covered with the soluble antigen of vaccinia virus moved at the same rate.

d. Rabbit papilloma virus. Sharp *et al.* (79) demonstrated that centrifugally purified rabbit papilloma virus migrated with a single peak at all pH values within the range of solubility and biological activity. Because of insolubility at its isoelectric point of pH 5.0, the mobility at this pH was measured by observing the migration of virus-covered collodion particles in the microscope. The point for the microscope determination fit the smooth pH-mobility curve determined by the moving boundary method (Fig. 18) in 0.1 ionic strength buffers.

e. Foot-and-mouth disease virus. Janssen (102) analyzed electrophoretically the vesicle lymph of foot-and-mouth diseased cattle in the Tiselius apparatus. Infectivity was found to be associated with a foreign protein having a peak in the region of the α_2- and β-globulins. Purification by precipitation or centrifugation yielded a nucleoprotein preparation which was electrophoretically homogeneous. Randrup (77, 103, 104) used the infectivity-sampling method to determine the mobilities of the complement-fixing antigens and the infectious entity of infected bovine vesicle lymph.

4. Bacterial Viruses

Todd (73) in 1927 followed the migration of a *Bacterium shiga* bacteriophage by means of infectivity measurements and concluded that the bacteriophage was negatively charged between pH 3.6 and pH 7.6. Using essentially the same method, Burnet and McKie (75) found that twenty different bacteriophage strains were negatively charged from pH 4 to pH 7. One phage, S13, migrated to the cathode under certain conditions. Putnam and associates (105–108) found the mobility of coliphage T6 to decrease from about -7.4×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ at pH 8.6 to about -3.8×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ at pH 5.1. Putnam (108) observed that the extraneous deoxyribonucleic acid present in crude phage preparations causes interaction effects between particles which might affect the mobility as measured by the moving boundary method. He suggests using the microscope method to avoid this complication. Putnam *et al.* (106) observed two boundaries in centrifugally purified preparations of T1 phage. Brinton (109) confirmed this and demonstrated that the slower component (-17.4×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$) was slightly more resistant to ultraviolet inactivation than the faster component (-23.2×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$) in 0.1 *M* veronal buffer, pH 7.6. Beumer *et al.* (110) have recently attempted to explain phage adsorption to dysentery bacteria by means of the colloid stability theory of Verwey and Overbeek (48). They used electrophoretic mobility data to calculate the ζ potentials of the phage and bacterium at various salt concentrations. From these, potential energy-distance curves were calculated which were only in partial agreement with the experimental results concerning the effect of cations on phage adsorption. This type of approach can be reasonably expected to explain only the nonspecific interaction between a phage and a bacterium, since electrophoresis is only capable of determining the average ζ potential over an entire surface. Specific phage adsorption leading to penetration and infection is probably determined only by a small portion of the phage and bacterial surfaces.

B. Bacteria

1. General

In contrast to the relative chemical simplicity and constancy of composition of the virus surface, the bacterial surface composition is tremendously variable. Almost any combination of protein, lipid, polysaccharide, and nucleic acid can occur on the surface in the form of cell walls, capsules, flagella, and filaments of various kinds. In addition, the metabolism of the cell causes the surface composition to change with physiological conditions. The electrophoretic mobility, being a function of *average* net surface charge density, reflects only the composite effect of surface components on the surface charge. As a consequence, the correlation of specific biological properties with relatively nonspecific mobility values is clear-cut only in special cases. Electrophoresis is used most advantageously in the analysis of heterogeneous populations of bacteria, the measurement of adsorption of charged substances onto the cell surface, and the following of changes in the surface as a function of time. This section will cover most of the recent advances and some of the older literature. Two recent comprehensive reviews of bacterial electrophoresis are those of Lerche (8) and James (10).

2. Mobility-Determining Surface Components

The surface of the bacterium determining its mobility is not only the cell wall, but also any other surface structure which moves kinetically with the bacterium. Some auxiliary surface structures are flagella, composed of pure protein (111), capsular filaments of proteinlike material (20, 24), capsular glutamyl polypeptide of *Bacillus subtilis* and *Bacillus anthracis* (112), the polysaccharide of pneumococcus (113), capsular cellulose fibrils of *Acetobacter xylinum* (114), both polypeptide and polysaccharide capsular material of *Bacillus megatherium* and other organisms (114). Capsular ribonucleic acid occurs (115), but capsular lipid has not yet been found.

The bacterial cell wall (22) contains protein, polysaccharide, and lipid components, in varying proportions. The total lipid content in per cent dry weight of gram-positive cell walls is about 1–2%, and for Gram-negative cell walls about 10–20%. The amounts of polysaccharide and amino sugar components vary from organism to organism, and there are no clear-cut differences between gram-negative and gram-positive cell walls. The amino acid constitution of cell walls also varies greatly among species and can make up from 15% to 95% of the cell wall. Cell walls from Gram-positive organisms lack some amino acids found in most proteins and in Gram-negative organisms; among the missing amino acids are phenylalanine and arginine.

The isoelectric points of bacteria are in general lower than those of viruses. This might be expected for several reasons: the presence of lipids, the occurrence of glutamic acid in capsules, and the frequent lack of arginine in cell walls.

3. Isoelectric Points and Stability

The low isoelectric points of bacteria are difficult to determine accurately because at low pH values capsular and cell wall material can be irreversibly altered. Evidence for modification of the bacterial surface at low pH has been obtained by Eggerth (116), Moyer (25), and Lowick and James (117). Moyer found that *Escherichia coli* cells washed at pH 2.5 and then rewashed at pH 6.9 with several changes of buffer had abnormal mobilities.

Because of the difficulty of measuring the mobility at low pH values, the variation in amount of amphoteric material with cultural conditions, and the paucity of amphoteric material in many bacterial strains, the isoelectric point is not a reliable characteristic value for bacteria. Winslow and Upton (118) and Burke and Gibson (119) found no correlation between isoelectric point and the gram property. Many species exhibit very little variation of mobility with pH (25, 15, 120). The isoelectric points that have been obtained for bacteria are nearly always quite low; some typical values from the literature are *Bacillus typhosus*, pH 3 (121); *Bacillus cereus*, pH 3.0 (122); pneumococci, pH 3 (123); *Micrococcus aureus*, pH 3 (45); *Aerobacter aerogenes*, pH 3.5 (118); *Erwinia amylovora*, pH 3.3, and *Phytomonas stewartii*, pH 3.5 (124); *Bacillus subtilis* and *Bacillus megatherium* spores, pH 2.0 (125).

The isoelectric point is related to the stability of bacterial suspensions. In dilute salt solutions ($<0.001\ N$) and in the absence of added protein, most bacteria agglutinate in the neighborhood of their isoelectric point. The region of agglutination for dilute salt solutions has been determined by Northrop and de Kruif (121, 126) and Shibley (127) to be the pH region where the ζ potential falls below about 15 millivolts. When foreign proteins are added to bacterial suspensions, they adsorb onto the surface and shift the isoelectric point toward that of the adsorbed protein. Because the isoelectric point of the foreign protein is nearly always higher than the bacterial isoelectric point and the adsorption is carried out on the alkaline side of the isoelectric point, the effect of protein adsorption is to reduce the charge density. Shibley (127) was able to demonstrate that specific immune serum reduced the charge on *Bacillus paratyphosus* and types I, II, and III pneumococcus to a significantly greater extent than did normal serum.

4. Factors Influencing Bacterial Mobility

The mobility of a given species or strain of bacterium depends upon the physiological condition of the cells and upon the treatment they receive after harvesting and before mobility is measured.

Physiological condition is determined by age of the culture and the culture medium employed. Some earlier investigators (128, 129) concluded that mobility is independent of age, but their cultures had already passed the period of physiological youth when mobility measurements were begun. Moyer (25) found that the mobility of *Escherichia coli* decreased about 20% after 1 hour's growth, remained low for 3 hours during the period of logarithmic growth, and then increased to the original value when the culture re-entered the lag phase. Watrous (130) found that the mobility of *Eberthella typhosa* increased with age of culture in two media and decreased with age in another medium. Stearns and Roepke (65) demonstrated a temporary decrease in mobility in growing cultures of *Brucella*. Barry and James (131, 132) measured the mobility of *Aerobacter aerogenes* throughout its growth cycle and found no variation. The question of mobility variation with age of culture can be standardized for comparative purposes by always using logarithmically growing cultures at a standard concentration.

The culture medium has a marked effect on mobility. Dyar (45) found higher mobilities for *Micrococcus aureus* cultivated in glucose medium than when cultivated in ordinary broth. The higher mobility was correlated with a higher lipid content. On the other hand, Moyer (25) and Tittsler and Berry (133) found the mobility of *Escherichia coli* to be independent of the type and concentration of the medium. Barry and James (132) observed that the mobility of *Aerobacter aerogenes* was the same in several synthetic media, broth, and agar. The strong dependence of capsule formation on cultural conditions probably accounts for many instances of mobility variation in different media.

Standard procedure before measuring mobility has been to wash the cells by two or three centrifugations and resuspensions in the same buffer as used for mobility measurement. Washing has been considered necessary for reproducible mobility measurements, presumably because of the removal of the culture medium or metabolic products adsorbed to the cell surface. Changes of mobility after washing have been demonstrated (25, 121). It has been generally overlooked that washing can remove loosely bound capsular material which is characteristic of a given bacterium and which can play an important role in determining its mobility and other surface properties. To avoid misrepresentation of mobility data, one should examine the cells in the electron microscope, for the presence or absence of capsules, flagella, filaments, etc., which might be removed by washing. Reproducible mobility values have been obtained without washing for encapsulated and nonencapsulated organisms by simply diluting the cells into the electrophoresis buffer (24).

With pathogenic organisms, it is desirable to sterilize the cells by heat or chemical treatment before measuring mobilities. In most instances, sterilization can be accomplished with little or no effect on the mobility by treatment with heat, formaldehyde, phenol, or mercuric chloride (120, 128, 134).

5. *Strain Specificity of Mobility*

The mobility of a given strain of bacteria is characteristic of that strain and remains constant over long periods of time through many subcultures if no genetic variation affecting mobility-determining structures occurs and if the mobility is always measured under the same conditions. *Escherichia coli* has been found to have a constant mobility when examined at various intervals over periods of up to five years (25, 134, 135). *Brucella* strains maintained their mobility through seventy-five consecutive tranfers (65). *Aerobacter aerogenes* mobility has been reported to remain constant over a period of several years (117).

There have been many attempts to correlate mobility with properties of bacteria used in taxonomic classification. Subgroups within species especially variants of the rough-smooth type, can often be distinguished by mobility measurements, but no general correlation exists between broad taxonomic groups and mobility. This can be seen (Table IV) from the mobilities of thirty-three different strains of bacteria of different family, genus, and species determined in phosphate buffer under approximately the same conditions of pH and ionic strength. Rough-smooth and virulent-avirulent variants of individual species often showed significant mobility differences, but no such differences existed among families, genera, or species. The mobilities of eighty-four strains of micrococcus vary continuously, with no obvious grouping, from 2.2 to 6.6 (μ/sec.)/(volt/cm.) (136). Harris (67) found no correlation between mobility and antibiotic tolerance, pigment production and lactose fermentation in *Paracolobactrum coliforme*, for bacteriophage resistance in *Xanthomonas pruni*, or for the Gram reaction (137).

A correlation of virulence with electrophoretic mobility has been claimed by some authors (121, 138–140) but disputed by others (141–145). It is possible that exotoxin produced by virulent bacteria and adsorbed to their surface could change their mobility; however, this effect might well be obscured by the washing used previous to mobility measurements in most investigations. Attempts to devise reliable clinical tests for pathogenicity of bacteria based on electrophoresis have failed.

The correlation between the ability of a bacterium to form rough or smooth colonies on solid media and its electrophoretic mobility has proved significant in a large number of cases. In the majority of instances the rough forms have a higher mobility than the smooth. Included are *Salmonella* and dysentery organisms (120, 146–150), *Escherichia coli* (20, 24, 25), *Brucella* (65), *Pasteurella tularensis* (66), *Mycobacterium tuberculosis* (151), *Mycobacterium leprae* (152), *Xanthomonas phaseoli* var. *sojenis* (153), and *Vibrio* strains (154). Two groups of workers claim to have observed higher mobilities for S variants than for R variants for type I *pneumococcus* (155) and tuberculosis bacilli (156). Dekker *et al.* (149) suggested that the colony morphology and electrophoretic behavior of some variants of *Salmonella typhi* were determined by their complement of Vi and IX antigens; Linton *et al.* (154) found that the degree of roughness of *Vibrio* strains could be ascertained more accurately by electrophoresis than by the agglutination method. Moyer (25) found different pH-mobility curves for R and S variants of *Escherichia coli*.

6. Effects of Surface-Modifying Agents on Bacterial Mobility

Changes in bacterial mobility caused by treatment with detergents, lipase, and trypsin have been described in Section IV. The effects of penicillin (157, 158), streptomycin (159), tyrothricin (158), sulfanilamide and

TABLE IV

MOBILITIES OF VARIOUS STRAINS OF BACTERIA IN PHOSPHATE BUFFER AT pH VALUES NEAR 7.0 AND IONIC STRENGTHS NEAR 0.02[a]

Organism	pH	Ionic strength	Mobility (μ/sec.)/(volt/cm.)	Reference
FAMILY PSEUDOMONADACEAE				
Pseudomonas aeruginosa	6.9	0.02	2.0	(51)
Pseudomonas aeruginosa	7.3	0.016	1.8	(128)
Spirillum volutans	6.9	0.02	2.3	(51)
FAMILY MICROCOCCACEAE				
Micrococcus pyogenes var. *aureus*	7.3	0.016	2.0	(128)
Micrococcus pyogenes var. *aureus*	7.4	0.026	1.8–5.7	(185)
Micrococcus pyogenes var. *aureus*	6.9	0.02	2.2	(51)
Micrococcus pyogenes var. *aureus*	6.9	0.02	2.5	(51)
Micrococcus citreus	7.3	0.016	2.0	(128)
Sarcina lutea	7.3	0.016	2.0	(128)
FAMILY LACTOBACTERIACEAE				
Diplococcus pneumoniae type I	7.2	0.026	4.3–4.5	(141)
Diplococcus pneumoniae type II	7.2	0.026	3.1–3.3	(141)
Diplococcus pneumoniae type III	7.2	0.026	5.7–6.4	(141)
FAMILY CORYNEBACTERIACEAE				
Corynebacterium diphtheriae virulent	7.2	0.026	1.9–3.9	(186)
Corynebacterium diphtheriae avirulent	7.2	0.026	3.9–5.1	(186)
Corynebacterium diphtheriae virulent	7.0	0.013	1.4–3.51	(144)
Corynebacterium diphtheriae avirulent	7.0	0.013	1.88–3.46	(144)
FAMILY ENTEROBACTERIACEAE				
Escherichia coli	7.3	0.016	2.9	(128)
Escherichia coli var. *communis* S	6.9	0.013	0.76	(25)
Escherichia coli var. *communis*	6.9	0.02	2.2	(51)
Escherichia coli B filamented	7.0	0.018	2.2	(24)
Escherichia coli B nonfilamented	7.0	0.018	3.6	(24)
Aerobacter aerogenes	7.0	0.013	2.36	(187)
Aerobacter cloaceae	7.3	0.016	1.3	(128)
Salmonella typhii	7.3	0.016	2.5	(128)
Salmonella gallinorum	6.9	0.013	0	(25)
FAMILY BACILLACEAE				
Bacillus subtilis	7.3	0.016	3.5	(128)
Bacillus megatherium	7.3	0.016	1.7	(128)
Bacillus pseudotetanicus	6.9	0.02	3.0	(51)
Bacillus pseudotetanicus (spores)	6.9	0.02	3.2	(51)
FAMILY MYCOBACTERIACEAE				
Mycobacterium phlei	6.9	0.02	2.8	(51)
Mycobacterium smegmatis	6.9	0.02	2.8	(51)
FAMILY MYXOCOCCACEAE				
Chrondrococcus columnaris	6.9	0.02	0.8	(51)
FAMILY SPIROCHAETACEAE				
Spirochaeta plicatilis	6.9	0.02	1.8	(51)

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[a] Taken in part from James (10).

derivatives (38), proflavine (160), crystal violet (117, 161), uranyl chloride (162), cetyl trimethyl ammonium bromide (163), acid dyes (164), quaternary ammonium disinfectants (165), ultraviolet irradiation (166), bacteriophage infection (167), X-irradiation (168, 169), and ribonuclease (170) have also been determined. Only a brief summary of the effects of some of these agents can be given here. In general, the mobilities of the bacteria are studied as functions of time of contact and of concentration of the agent, under different physiological conditions. With few exceptions, treatment of the surface reduces its negative mobility.

McQuillen (157) reported that the mobility of washed suspensions of *Staphylococcus aureus* is unchanged in the presence of penicillin, indicating that penicillin is not attached to the surface. However, the negative mobility was reduced for organisms grown in dilute broth containing penicillin and subsequently washed, the magnitude of the reduction depending upon the concentration of the penicillin. The scatter of individual mobilities was also increased. Similar effects were noted with *Escherichia coli*. Streptomycin is rapidly adsorbed to the surface of *Escherichia coli* in the presence of dilute broth, reducing the mobility (159). However, this mobility reduction can be completely reversed by washing.

James and Barry (160) observed that the mobility of *Aerobacter aerogenes* decreased as the concentration of proflavine was increased. The mobility distribution of organisms grown in the presence of proflavine became more inhomogeneous and was shifted toward lower mobility values. The altered distribution was maintained during growth in the absence of the drug and also in cultures derived from a single organism.

Lowick and James (117, 161) investigated the effect of crystal violet on *Aerobacter aerogenes*. When the dye was adsorbed onto cells, it caused the mobility to decrease, but this effect was completely reversed by washing. Changes in mobility not reversible by washing occurred only during cell multiplication. The pH-mobility curves of normal cells and dye-trained cells were different. From consideration of mobility changes brought about by treatment with anionic and cationic detergents, organic solvents, and lipase, these authors concluded that the surfaces of cells trained to grow in crystal violet had more lipid than the surfaces of normal cells.

The detergent cetyl trimethyl ammonium bromide decreases the charge of *Escherichia coli*, and the charge of two gram-positive organisms first rose and then fell as the concentration of this detergent was increased (163). Uranyl chloride decreased the charge of *Escherichia coli* and *Staphylococcus aureus* and increased the charge of *Streptococcus faecalis* (162). Washing in phosphate buffer reversed the effect. Harris (164) demonstrated that bacterial binding of acid dyes was stronger when the negative charge of the cells was decreased by lowering the pH or increasing the salt concentration.

C. Erythrocytes

Red blood cells deserve attention because of the important reactions which take place at their surface, the constancy of their electrophoretic properties, and the ease with which their mobility can be measured.

1. Surface Structure

The human erythrocyte is a biconcave disk about 7.8μ in diameter. From an electron microscope examination, Hillier and Hoffman (23) have concluded that the cell membrane is about 50 Å. thick and is made up of an inner layer of fibers 20 Å. in diameter and an outer layer of plaques about 30 Å. thick and 200 Å. in diameter. The inner fibers are oriented perpendicularly to the radius. The plaques might also have a fibrous structure and can be removed from the inner fibers by lipid solvents, which apparently do not change the plaque structure. There are pores separating the plaques which are presumably lined with lipid. The plaques are probably identical with a fraction, obtained from blood cells by ether extraction (171), consisting of long, thin rods composed of a lipid-carbohydrate-protein complex called stromin. Hemoglobin might be associated with stromin (172).

2. Mobility and Charge Density

The electrokinetic properties of erythrocytes from eleven different mammals are summarized in Table V (4). The mobility is constant for a given species but varies among species in a way unrelated to the phylogenetic classification of mammals. Human red cell mobility in $M/15$, pH 7.3 phosphate buffer has a value of 1.3 (μ/sec.)/(volt/cm.), which is independent of race, age, sex, blood group, pregnancy, or anemia. Its universal availability and constant mobility make the human red cell an ideal particle for the calibration of microscope electrophoresis apparatuses. The surface charge density, σ, in column three of Table V is calculated using equations (2) and (8). In column four, σ' is corrected for the finite size of the ions in the double layer by dividing σ' by $1 + \kappa a_c$, where $a_c = 2.5$ Å. is assumed to be the average radius of the counterions (4). Equations (2) and (8) are valid only for large, smooth particles where $\kappa a > 100$. If the effective value for the average radius of curvature of the blood cell surface is, for example, 20 Å. rather than infinity, both ζ and σ would be about twice as great.

The dependence of the mobility of erythrocytes on ionic strength and pH has been determined for human cells by Furchgott and Ponder (173)

TABLE V

ELECTROKINETIC PROPERTIES OF MAMMALIAN RED BLOOD CELLS IN $M/15$ pH 7.3 PHOSPHATE BUFFER[a]

Animal	Mobility (μ/sec.)	ζ (volts)	σ Uncorrected (E.S.U./cm.^{2})	σ' Corrected (E.S.U./cm.^{2})
Rabbit	0.55	0.0070	1890	1410
Sloth	0.97	0.0124	3330	2480
Pig	0.98	0.0125	3360	2500
Opossum	1.07	0.0137	3680	2740
Guinea pig	1.11	0.0142	3780	2820
Man	1.31	0.0168	4500	3350
Rhesus monkey	1.33	0.0170	4570	3400
Cat	1.39	0.0178	4780	3560
Mouse	1.40	0.0179	4800	3580
Rat	1.45	0.0186	4980	3710
Dog	1.65	0.0211	5660	4220

[a] From Abramson *et al.* (4).

and for guinea pig cells by Bateman and Zellner (174). The mobility-ionic strength data of Furchgott and Ponder are plotted in Fig. 19, ionic strength being plotted as $1/\kappa$. The ionic strength was varied by diluting an NaCl-phosphate buffer solution at pH 7.3 and ionic strength 0.172 with a 5.4% glucose solution to maintain isotonicity. Since NaCl contributed 90% of

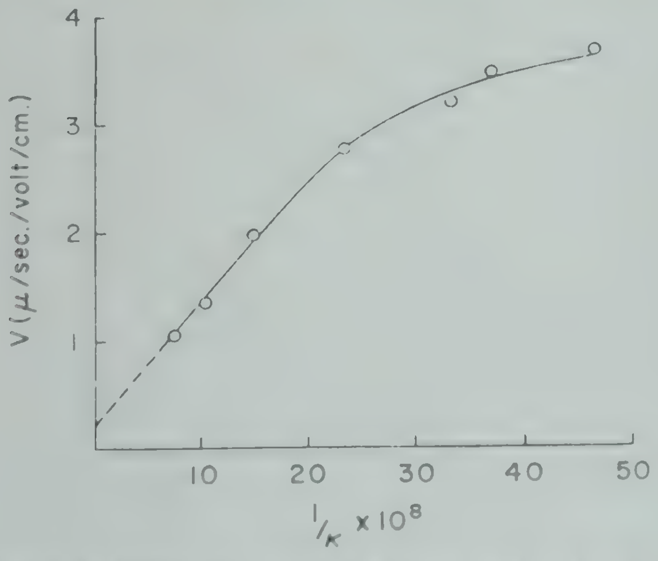

FIG. 19. Mobility of human red cells as a function of $1/\kappa$. All mobilities corrected to the viscosity of water. From Furchgott and Ponder (173).

the ionic strength, the solution behaved essentially as a uni-univalent electrolyte. At the highest dilution, the buffer concentration was so low that the pH value dropped to pH 6.85; however, the mobility is almost independent of pH in this region. Equation (7) valid for $\kappa a > 100$ corrected according to Gorin (Abramson *et al.*, 4) for the finite size of the counterions takes the form

$$u = \frac{\sigma}{\eta}\left(\frac{1}{\kappa} + a_i\right) \tag{23}$$

If σ were constant, a straight line with slope σ/η and intercept $(\sigma/\eta)\ a_i$ would be obtained for the plot of u vs. $1/\kappa$. On the contrary, a curved line is obtained. Although Furchgott and Ponder (173), and Abramson *et al.* (4) state that the decrease in slope could be the result of "a change from a smooth to a bumpy surface," they choose to interpret the curve as meaning a change in σ at lower ionic strengths. In addition, the linearity of the curve at ionic strengths greater than about 0.02 is given as evidence that in this region the red cell behaves as though it were a large particle with a smooth surface. The extrapolated intercept at $1/\kappa = 0$ is divided by the slope at high ionic strength to obtain $a_i = 1.8$ Å. This value for a_i is of the correct order of magnitude and it is implied that this result makes the correction for finite ion size plausible.

Bateman and Zellner (174) have calculated σ for guinea pig red cells using the usual combination of equations (2) and (8), both of which are valid only for large, smooth particles. They, too, conclude that σ decreases at low ionic strengths, and attribute this to changes in the character and position of ionizable groups in the cell membrane. They were able to plot the σ-ionic strength data of Furchgott and Ponder (173), Brody (175), and their own data in the form of Langmuir adsorption isotherms.

The σ-ionic strength data on erythrocytes are subject, however, to an alternative interpretation. If the radius of curvature, a, is not that of the whole particle but is instead the average radius of curvature of the surface itself, κa can be much less than 100 and equations (2), (8), and (23) are no longer valid. Electron microscope data cited above support the hypothesis that the red cell surface is not smooth. Since it is difficult to determine the effective radius of curvature of the surface, we have assumed values of a equal to 20 Å., 40 Å., and 100 Å., and have compared σ calculated using these values of a with σ calculated assuming a is very large. For the data of Furchgott and Ponder, κa can vary from 0.4 to 13.3

For these values of κa, the use of Henry's equation is essential for the calculation of the ζ potential, and the Debye-Hückel formulations relating surface charge density to ζ also take different forms. Assuming that the fine structure of the red cell surface is spherical in shape, and that the

Debye-Hückel approximation holds, we can combine equations (4) and (5) to obtain σ in terms of u. Introducing Gorin's correction for the finite size of the counterions, we obtain

$$u = \frac{4\sigma a f(\kappa a)\,(1 + \kappa a_i)}{6\eta\,(1 + \kappa a)} \tag{24}$$

This equation can be rearranged algebraically into the form

$$\frac{6u(1 + \kappa a)}{4\kappa a\, f(\kappa a)} = \frac{\sigma}{\eta}\left(\frac{1}{\kappa} + a_i\right) \tag{25}$$

which is the equivalent of equation (23), taking into account that κa is in the region $1/10 < \kappa a < 100$. If the quantity on the left is plotted against $1/\kappa$, a straight line with slope σ/η and intercept $(\sigma/\eta)a_i$ should be obtained if σ is constant. This plot is shown in Fig. 20 for the data of Furchgott and Ponder. The smaller the radius of curvature that one assumes, the more nearly the plot approaches a straight line. At $a = 20$ Å., the points fit very closely to a straight line, and at $a = 40$ Å., the curve is not significantly different from a straight line. The intercepts at $1/\kappa = 0$, from which a_i is determined, are positive, negative, or zero. Figure 20 shows that the mobility data on blood cells can be made consistent with the assumptions that the net surface charge density is constant with changing ionic strength, the surface is not smooth, and the correction for finite counterion size is not applicable.

In addition to inexact knowledge of the radius, there are many other uncertainties involved in calculating σ when κa is in the intermediate region of 1/10 to 100. The form of the equation relating σ and u is different for spheres, cylinders, and disks; equations for other shapes have not been derived. For ζ potentials above 25 millivolts, and for unsymmetrical electrolytes the Debye-Hückel approximation no longer holds and more exact equations must be employed. Corrections for the relaxation and surface conductance effects must also be applied (7). For the data of Furchgott and Ponder the maximum correction to the charge necessitated because ζ is greater than 25 millivolts (7) is of the order of magnitude of 15 % because the electrolyte is essentially uni-univalent and $e\zeta/kT$ has a maximum value of 2.3 at the lowest ionic strength. In any case, the correction would *increase* the calculated charge density at low ionic strengths. This would have the effect of straightening the curves in Fig. 20 at somewhat higher values of the assumed radius of curvature.

If instead of a spherically shaped surface roughness, one assumes that the mobility-determining structures of the red cell surface are cylinders of radius a oriented either perpendicularly or at random to the field, the curves in Fig. 20 are only slightly altered. A scheme for the reduction of

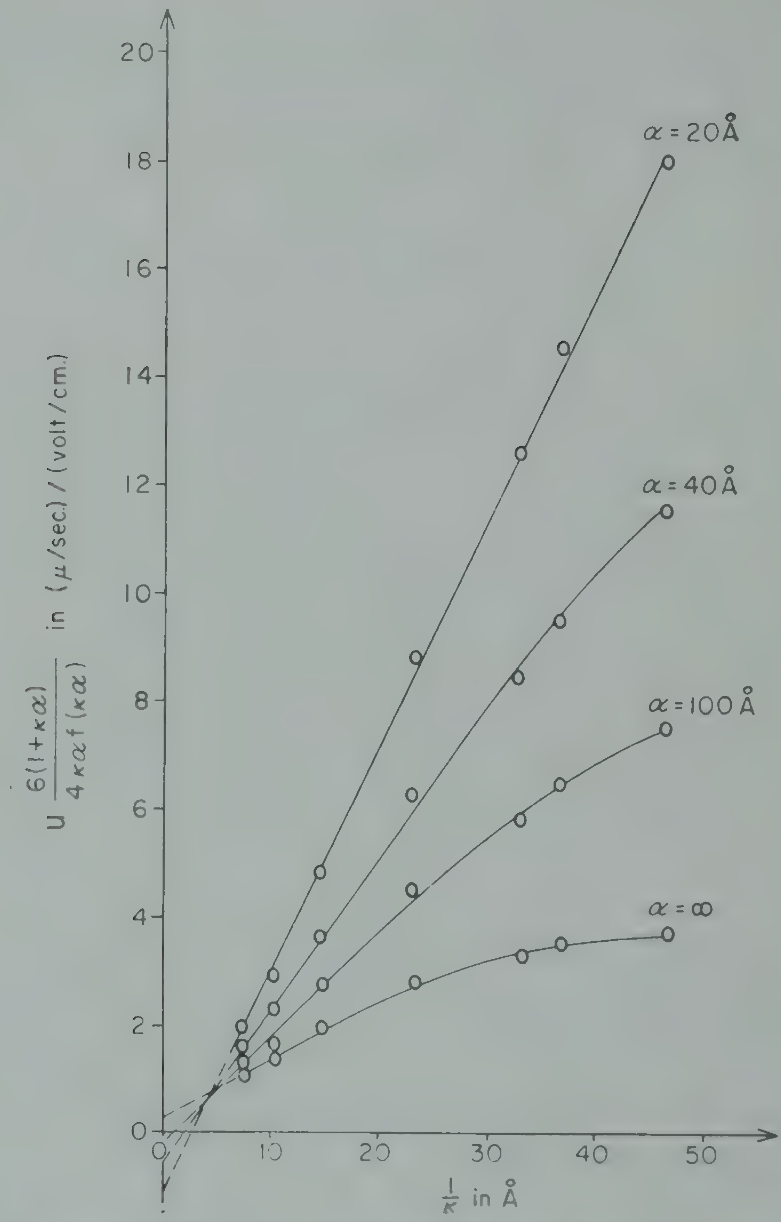

Fig. 20. Mobility data of Furchgott and Ponder (173) plotted according to equation (25) as a function of $1/\kappa$ for different values of a, the radius of curvature of the blood cell surface. The curve for $a = \infty$ is identical to the curve of Fig. 19.

the net negative charge density of the red cell surface by virus attachment, proposed by Burnet (175a), assumes that the charged elements are chains protruding from the surface. This hypothesis is not inconsistent with the above interpretation of the mobility data.

It can be seen from Fig. 20 and equation (25) that σ is very sensitive to the proper choice of radius of curvature, much more so than the ζ potential [equation (4)]. Conclusions concerning the nature of the surface arrived at from consideration of charge density values and their variation with salt concentration must be accepted with great reservation as long as the radius of curvature of the surface is unknown.

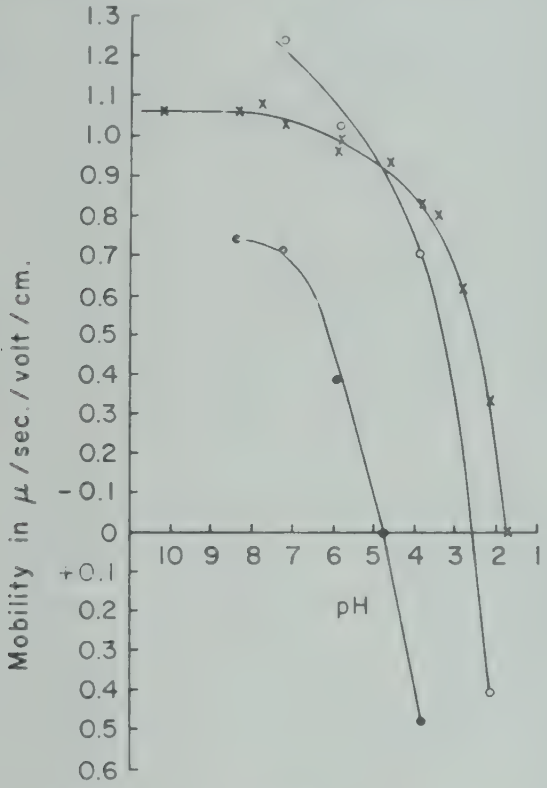

FIG. 21. pH-Mobility curves at ionic strength 0.172 for human red cells (×), for the lipid extract of the cells (○), and for the stroma protein of the cells (●). From Furchgott and Ponder (173).

The pH-mobility curves for human red cells, lipid extracted from the cells, and the stroma protein of the cells are shown in Fig. 21. The isoelectric point of the whole red cells is very low, as is the case for most cells. At low pH values, the cell surface is damaged and the mobility is altered, perhaps because of adsorbed protein. To obtain their curve, Furchgott and Ponder measured the mobility within 30 seconds of lowering the pH, while the mobility was still unchanged. Electrokinetically, the whole surface is more like the extracted lipid than the protein, but this is not conclusive proof that the mobility-determining surface structures are lipid.

3. Treatments Which Do Not Affect Erythrocyte Mobility

The red cell surface is remarkable in that many treatments which might be expected to change its mobility do not. The mobility of red cells has been shown to be unaffected by the presence of many proteins, including egg albumin, crystalbumin, gelatin, casein, hemoglobin, serum globulin, and fibrinogen (4). Lysis of red cells by saponin, water, freezing and thawing, chloroform, or specific immune serum in the presence of complement, can occur without significantly affecting the mobility. This could be either because only certain "keyspots" (4) comprising a small portion of the surface need be affected to produce lysis, or because the mobility-determining structures of the red cell are entirely unaffected by lytic agents.

4. Treatments Which Affect Erythrocyte Mobility

The mobility of red cells is significantly reduced by treatment with viruses of the mumps-influenza group and by the receptor-destroying enzyme (RDE) of *Vibrio cholerae*. The discovery of this effect by Hanig (49) has already been discussed in Section IV, C, 1. In extensions of Hanig's work, Ada and Stone (176, 177) found that the amount of mobility-lowering by the mumps-influenza group of viruses and RDE closely resembled the "receptor gradient" of Burnet *et al.* (178). Two exceptions were the viruses of swine influenza and Newcastle disease, which lowered the mobility more than would be expected from their position in the receptor gradient. Stone and Ada (179), on the basis of more precise data, postulated that most of the viruses fall within one group with a single enzyme action on a single red cell substrate. Newcastle disease virus, swine influenza virus, and RDE of *Vibrio cholerae* were thought to have an additional action belonging to the same enzyme but acting on a different substrate.

D. Spermatozoa

The electrophoretic migration of sperm cells is especially interesting, although the literature on this subject comprises only a few dozen papers in all. Mudd *et al.* (180) observed that after the current had flowed for a short time, rabbit and sea urchin sperm migrated tail foremost. This orientation is also true of human sperm (181). There are only a few examples of large particles being oriented by the electric field; the only others which have come to our attention were flagellated bacteria (51, 182). Both Mudd *et al.* and Abramson (4) conclude that the sperm tail must have a higher ζ potential than the head, which is the most reasonable hypothesis. Gordon (183) suggests, in addition to the latter hypothesis, that the tail might have a larger *volume* charge density; this is incompatible with electrophoretic theory and experiment which show that only the surface charge density determines mobility.

Mudd *et al.* studied the effect of specific antisera adsorbed on human, ram, bull, and rat sperm and found that homologous sera reduced the mobility to a greater extent than did heterologous sera. This effect is analogous to that observed for bacteria (127).

Electrophoresis has also been used to separate sperm into two populations, one of which allegedly produces female offspring (anode migrating) and the other male offspring (cathode migrating) (184). Gordon (183) has recently repeated this work and summarized the literature. We shall confine ourselves to a discussion of his paper. Rabbit semen was diluted 1:20 with a glycine buffer at pH 7.1 and subjected to electrophoresis for 40–50 minutes. The orientation was always with the tail in the direction of motion. Approximately equal numbers migrated toward the cathode and the anode. Samples of 0.5–0.7 ml. were withdrawn from one pole of the electrophoresis cell, which could be made either the anode or the cathode. A comparison of the sex of offspring with the electric pole of isolation is shown in Table VI. There is a significant correlation between pole of isolation and sex of offspring; the P value for the total data is <0.001 and for the coded data <0.008.

TABLE VI

CONTINGENCY TABLES OF SEX OF OFFSPRING COMPARED WITH ELECTRIC POLE OF ISOLATION[a]

Pole	Total data		Coded data	
	Female	Male	Female	Male
Anode	62	25	50	22
Cathode	29	51	27	31

[a] From Gordon (183).

Critical scientists will find the paper by Gordon disappointing. It is unfortunate that no description of the cell or of the isolation technique is given. The mobility distribution of the isolated cathode and anode populations does not seem to have been determined.

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Chapter 11

Applications of Zone Electrophoresis*

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I. General Experimental Conditions

The electrophoretic separation of low-molecular substances can be accomplished only by methods in which the bands traveling at different rates in the electrical field are stabilized and do not become blurred by diffusion, i.e. methods employing supporting media. Before presenting a list of the applications of this "zone electrophoresis" (pherography), which in view of the very large number of publications can be only a very selective one, the experimental conditions may first be described in brief. A more detailed description of the various methods is presented in Chapters 5 and 6 of this book.

A. *Supporting Media*

Porous papers, fabrics, premixed powders, gels, or—for ultramicro amounts—threads are used as supports for zone electrophoresis.

1. Papers and Fabrics

The material in widest use is filter paper made of very pure cotton fibers, such as is employed extensively in various countries for paper chromatography (manufactured by Whatman, Schleicher and Schüll, Macherey, Nagel and Co., Munktell, and others). Several types of all these brands are sold, of different thickness up to pasteboards several millimeters thick which permit the separation of up to 100 mg. of material.

Almost all brands of paper contain water-soluble impurities. Water flowing through a 1-square-meter area of paper by capillary action may elute up to 50 mg. of substances (1) which after the hydrolysis usual for peptides yield the ninhydrin-positive spots characteristic for α-amino acids upon paper chromatography or electrophoresis (2, 3). It is therefore advisable to wash the papers by a thorough process of percolation of water or of dilute acid followed by water containing 8-hydroxyquinoline for the removal of heavy metals. Fabrics of polyvinyl chloride [Rhovyl paper of Schleicher and Schüll (4)], of glass fibers (5), or of silk (6) have an advantage as supports over paper in that the localization of the separated substances can be carried out by color reactions in which the paper fiber is destroyed or takes part in the reaction. Examples are given in the discussion on separation of sugars (page 511). According to the author's experience, the disadvantages of these fabrics are smaller capacity, greater porosity and consequently greater diffusion, and pronounced electroosmosis.

2. Powders

The following powders have been used successfully: potato starch (7), "soluble" starch, cellulose powder (e.g. cf (8)), or glass powder, which is prepared most expediently by grinding glass wool under water and fractional sedimentation (9). Of course, the powders listed above do not exhaust the list of possibilities, since in principle one may use any pulverizable, fairly water-insoluble material, for example, plastics [polyvinyl chloride (10)] or minerals [Hyflo-Super Cel (11); asbestos (12)]. Adsorption of the substances on the support and electroosmosis play the principal roles in the determination of the suitability of supporting media.

The powders are made into a thick, homogeneous paste with the buffer, applied as a layer to the base of the trough (2–10 mm.), and then pressed thoroughly dry with filter paper. In columns, a thin suspension is poured evenly, as in the filling of a chromatographic column, and the solid material is then allowed to settle firmly. The same procedure is used in loading the continuously operating instruments for preparative electrophoresis described in Chapter 6.

3. Gels

In the first zone electrophoretic separation, that of amino acids carried out by Consden and co-workers (13), *silica gel* was employed as the supporting layer. To give an example, 28 ml. of a 4.3 N solution of sodium silicate was diluted to 350 ml. with water, neutralized with 1.6 M phosphoric acid, and poured into the shallow electrophoresis trough where it solidified into a gel. In the center, a slab of 1-cm. width was then cut out and filled with the mixture of sodium silicate and the substance to be analyzed, prepared in the same way.

Even older is the use of *agar gel* [first reported in 1923 by Kendall and Crittenden (14)] for inorganic ions. This supporting medium later proved to be of value also for organic substances [e.g. see (15)]; it is poured as a 1% warm solution, which upon cooling solidifies into a mechanically firm mass. However, in acid solution the coagulation does not take place. Recently, a *starch gel* obtained by heating starch with water has been used successfully, in particular for protein electrophoresis (16).

For the separation of proteins, a finely porous rubber *sponge* has been recommended as the supporting medium (17).

4. Threads

In this regard, the ultramicro method of Edström (18), which permits the separation of amounts of nucleotides down to 10^{-10} gm., is a representative example. A 1- to 2-cm. long rayon fiber was prepared from cuprammonium viscose, swelled in alkali, washed with water, and saturated for 24 hours in a citrate buffer of pH 3.6, made viscous with glycerol and glucose. The saturated fiber was covered with a buffer containing paste at both ends and stretched across a hollow space in a thick glass plate. A hydrochloric acid hydrolyzate of ribonucleic acid was added at an arbitrary point, the depression filled with paraffin, the chamber closed with a quartz disk, and the potential applied from microplatinum electrodes with a voltage of 1.2 volts per centimeter. After 2 hours, adenine, guanine, cytidylic acid, and uridylic acid were definitely separated along 1 mm. of the fiber, as shown by ultraviolet photography. Photometric measurements permit quantitative estimates of sufficient accuracy so that differences in the nucleic acid composition of single cells may be detected.

Finally, the arrangement for zone electrophoresis according to Svensson (19) should be mentioned, in which the stabilization is achieved by a density gradient without supporting media. Electrophoretic separations may also be carried out without supporting media, on a microscope slide in an extremely thin (0.01–10μ) layer of fluid (19a).

B. Some Comments Concerning Technique

Zone electrophoresis may be used as a qualitative or quantitative analytical or preparative method, depending on the amount of substance which is applied. For analytical purposes, the papers and fabrics mentioned under Section I, A, 1 are usually employed, and they permit a micropreparative work-up as well, if greater thicknesses are used. For the handling of amounts of 100 mg. or more, the supporting layers, columns, and continuous arrangements come into play. Since in the author's experience high-voltage electrophoresis on paper (50–100 volts per centimeter) with cooling (1, 20–22) provides the sharpest separations of low-molecular substances, the surest preparative procedure for the collection of substances which differ only slightly in their migration consists in a succession of several (up to fifty) "analytical" separations of the mixture applied in the form of a streak and in elution of the bands. This is not too impractical an arrangement since each run generally lasts only 1–2 hours and since two samples can be placed one behind the other on one sheet at a suitable distance from each other.

Some of the errors which, despite the simplicity of paper electrophoresis, will lead to unsatisfactory results may be mentioned briefly (1). In addition to impurities in the paper and the use of unsuitable buffers, these are:

(1) Scratching the surface of the paper. The capillary pipette used for the application of the solution to be separated must have a rounded tip. A tip with sharp edges, especially if brought down on the paper firmly, tears small shreds out of the paper texture along its trail, which act as tiny reservoirs for the substances and cause them to deposit as fine parallel streaks during migration.

(2) Application of alien solvents. Solvents with electrical properties different from the buffer lead to a distortion of the electrical field and therefore to a lengthwise shrinkage of the separated bands.

(3) Excessive amounts of salt. Even though paper electrophoresis constitutes a convenient method for the removal of reasonable amounts of salt, excessive amounts (e.g. more than 20 mg. sodium chloride in the application band) have a disturbing influence. The consequent reduction of the electrical field may lead to a pronounced slowing of the migration of substances.

Crestfield and Allen (23) have pointed out the undesirability of marking the sheets of paper with pencil. Such marks of graphite in the electrical field become secondary electrodes at which electrochemical reactions may take place.

For other supporting media, requirements similar to those for paper electrophoresis as to purity—particularly the absence of heavy metals and of large amounts of salt—capacity, and so on, must be met.

C. Detection and Evaluation

1. General Detection Methods

Many of the substances to be described are colorless and therefore cannot be localized directly. They may be recognized by physical, chemical, or biological means. The reactions used for detection, which almost without exception were elaborated during the development of paper chromatographic techniques, are summarized completely in the appropriate handbooks and the progress reports in *Analytical Chemistry*. For this reason, they are not discussed in detail here. For the separation of materials treated in the following section, the customary methods of detection are described briefly. In this section, we may enumerate briefly the generally applicable principles, but it will be impossible to cite the extensive literature sources one by one.

Paper or fabrics are examined directly by one of the methods to be described below. The surface of powder or gel layers can be placed in intimate contact with a piece of filter paper and thus "transferred" on the latter and judged by means of this copy. In column electrophoresis, direct or indirect observation of the separated zones is sometimes possible. Otherwise the column is cut after electrophoresis and the portions are eluted, or the materials are eluted in fractionated form from the column in the manner of a flow chromatogram. In continuous set-ups, the separated substances are collected in individual vessels and can be determined there in solution or as drops on paper by a spot test.

a. Physical methods. Fluorescent substances are detected in ultraviolet light. Others which absorb in the ultraviolet range, for example aromatic and heterocyclic compounds such as purines and pyrimidines, are recognized as dark spots by *quenching* of either the weak fluorescence of the paper itself or of the fluorescence of strongly fluorescent substances, such as fluorescein or acridine, which can be sprayed on the paper. The light source is a filtered mercury high pressure lamp which furnishes fairly pure light at 254 mμ.

Radioactive materials are recognized by the blackening of a silver bromide film (X-ray film) placed in close contact with the paper for a fairly long time and then developed and fixed (autoradiography) or by counting the radioactivity in the paper with a Geiger counter. The substances to be analyzed may be made radioactive either by prior incorporation of isotopes, or by subsequent reaction with radioactive reagents followed by removal of excess reagent, or by bombardment with neutrons [induced radioactivity in compounds containing S, Br, Cl, or P (24)].

b. Chemical methods. Substances can be made visible by a specific reaction with dyes or with colored substances, by the formation of colored products by means of special color reactions, by the formation of fluorescent substances on the supporting medium, by decoloration of sprayed-on colored reagents, or by pH or redox indicators.

c. Biological methods. The conversion of the very specific enzyme reactions into spray or dip tests for substances which have been separated by paper chromatography or electrophoresis is very promising but has been used only rarely until now. Frequently, however, growth-promoting activity (vitamins) or growth-inhibiting activity (antibiotics) is determined by placing the strips on agar plates inoculated with bacteria (bioautography).

2. Quantitative Evaluation

This topic can also be treated only very briefly since these methods too are described in detail in the treatises on paper chromatography. The most precise results are obtained by the complete elution of the substances after the application of one of the detection methods enumerated under Section I, C, 1 and subsequent assay of concentration in the solution by means of one of the numerous standard methods. As in paper chromatography, methods have been developed also for direct determination on paper strips, and several of these will be described in the discussion of the appropriate classes of substances.

D. *High-Voltage Electrophoresis*

Electrophoretic separation, predominantly on paper but also on other supporting media, is achieved more rapidly if the field intensity is increased. In addition to a saving of time, the application of higher field intensities (20–100 volts per centimeter) has the advantage of sharper separations since the interfering diffusion of the substances in the supporting medium is of less significance. However, because of the increased current intensity and heat evolved, cooling becomes necessary. This exerts a further favorable influence on the sharpness of the separated bands, in view of the temperature dependence of diffusion, which is less pronounced at lower temperatures. Low temperatures also permit the analysis and separation of unstable substances, frequently encountered in biochemical work (energy-rich phosphates, proteins, etc.). High voltage, therefore, is primarily a problem of cooling.

In the simplest arrangement for paper electrophoresis, consisting of a paper strip stretched in a moist chamber (25), cooling to 20–30°C. is achieved by the evaporation of the electrolyte solution. But this suffices only up to 10–20 volts per centimeter, and more effective cooling is required at higher voltages. Two methods may be used to achieve this.

(1) The immersion of the strip, saturated with buffer, into a larger volume of water-immiscible insulating fluid. This principle operates in the methods of Michl (1951) (20) and Markham and Smith (1952) (26). In the former, the strip moistened with buffer is suspended between an upper and a lower electrode compartment in a large container filled with toluene (Fig. 1). In the latter, the strip is led between the two electrode compartments through a trough filled with carbon tetrachloride. In order to obtain the longest possible migration path, the paper is stretched in the form of a V with the aid of a celluloid plate placed in the center.

Both arrangements have proved satisfactory. Of course, they permit only the separation of substances which are insoluble in the cooling solutions; they cannot be used for electrophoresis in supporting layers which are not interwoven or matted.

(2) By application of the paper or the supporting medium on cooled surfaces. This principle is applied in several instruments. It was first proposed for paper electrophoresis at low voltage by Kraus and Smith (27), who placed the strip on an insulated copper block. A short time later, an arrangement was described, once again by Michl (20), in which the paper strip is pressed between two cooling surfaces of aluminum covered with

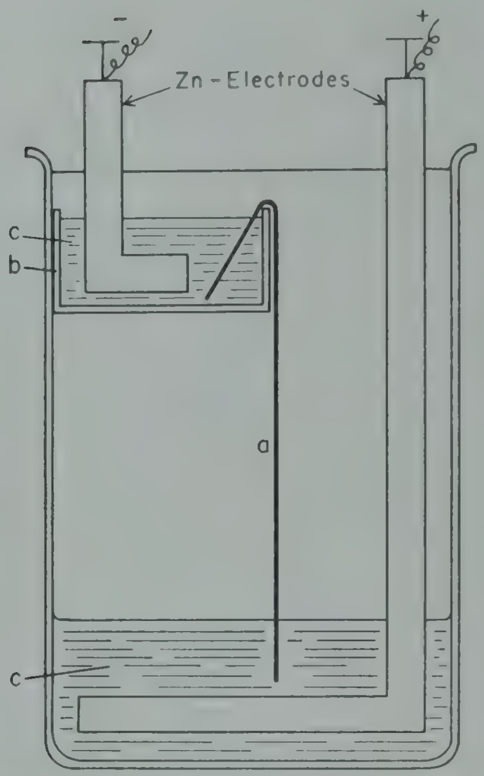

FIG. 1. High-voltage electrophoresis apparatus of Michl (20). *a*. Paper strip; *b*. electrode vessel; *c*. electrolyte.

insulating layers of nylon primer. Each cooling surface is part of a cooling chamber perfused with water. The paper strip in this arrangement is not in direct contact with the buffer solutions of the electrode vessels, a cellophane membrane being inserted between the strip and the paper or linen wicks which dip into the electrode solutions.

This membrane principle was originated by R. Weber (28), who observed that the electrophoretically separated spots remain more compact if most of the freely absorbed buffer is squeezed out of the paper strips. In such "dry" systems, diffusion is greatly diminished. These papers with a low water content should not have their ends immersed in the buffer vessels of the electrodes since they would then reabsorb fluid. They are therefore separated by cellophane membranes from paper wicks which dip into the electrode vessels. In this way, electroosmosis as well as fluid absorbtion is largely restrained.

The arrangements described by Foster (29) and Gross (30) work, not on this principle, but with cooling; they also employ cooling surfaces made of metal which are insulate from the paper by glass or polyethylene. In both instruments cooling is carried out with tap water. Field intensities of 50–80 volts per centimeter can be used.

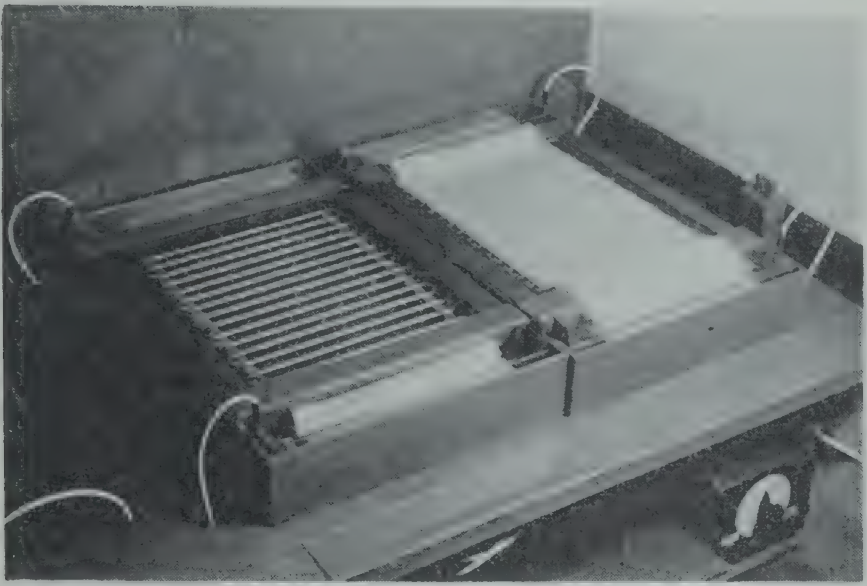

FIG. 2. Apparatus according to Wieland and Pfleiderer (22). At right, cooling chamber with a sheet of paper; in the foreground, the two cellophane-covered wicks which lead into the electrode vessels.

While the above arrangements incorporate most of the simple principles of successful high-voltage electrophoresis, they do not permit cooling to 0–10°C.; special cooling systems must be employed. The instruments described by Werner and Westphal (21) and by Wieland and Pfleiderer (22) make use of such systems. In the former, the cooling surface consists of a plastic chamber transversed by cooling brine; in the latter, which permits the use of 35-cm. wide strips, a glass plate is employed because of its better heat conductivity. Cooling is carried out by a spiral heat exchanger from a refrigeration unit, which is immersed in the methanol-water mixture filling the chamber* (Fig. 2). In both instruments, the current is applied through cellophane membranes.

The glass cooling surface also permits the use of thicker supporting layers at higher voltages. Thus, many enzymes can be separated electrophoretically in a 0.5-cm. thick starch layer at 20–30 volts per centimeter and can then be obtained pure by elution in a micropreparative procedure (1a).

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* "Pherograph-Frankfurt," manufacturer, L. Hormuth; owner, W. E. Vetter; Heidelberg-Wiesloch, West Germany.

II. Electrophoretically Separable Mixtures

A. Amino Acids

For this class of compounds, the electrophoretic separation in silica gel (13) and filter paper (25) was first elaborated. In silica gel, the following separations were achieved: 30 mg. of a hydrolyzate of wool into acidic, neutral, and basic fractions in phosphate buffer at pH 7; glycine and serine in borate buffer at pH 9.7; methionine and alanine in borate at pH 9.4; glutamic acid from aspartic acid in acetate buffer at pH 7; lysine from histidine in NaCl solution at pH 6.6. Wide experience has been gained on *paper,* starting with our first experiments (25) by way of the extensive studies of Durrum (31) to the application of high voltages to well-squeezed-

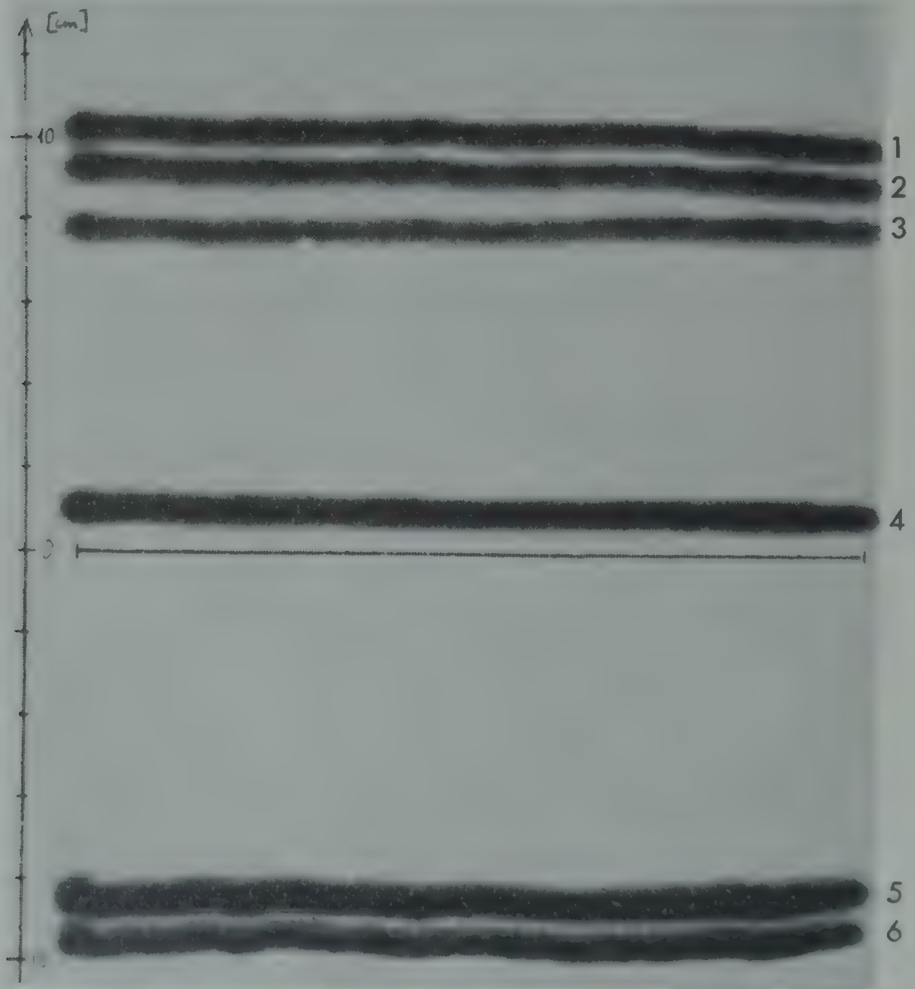


Fig. 3. Paper electrophoretic separation of basic, neutral, and acidic amino acids at pH 6 (according to H. Bodemann). 1. Lysine; 2. Arginine; 3. Histidine; 4. Alanine; 5. Glutamic acid; 6. Aspartic acid.

out sheets of paper on cooled surfaces (1, 20–22). Such a separation of a mixture of amino acids into aspartic acid, glutamic acid, a neutral fraction, histidine, arginine, and lysine in pyridine-glacial acetic acid-water at pH 6.5 (20) is shown in Fig. 3. The separation of neutral, basic, and acidic amino acids at pH 1.9 in glacial acetic acid-formic acid-water (32), followed by visualization with ninhydrin, is shown in Fig. 4.

A complex mixture with thirteen components could be separated by Durrum (31) by means of *two-dimensional* electrophoresis (first dimension in 0.2 *N* aqueous NH_4OH, pH 11.3, 120 minutes; second dimension in 1 *M* acetic acid, pH 3.2, 30 volts per centimeter, 180 minutes) (Fig. 5).

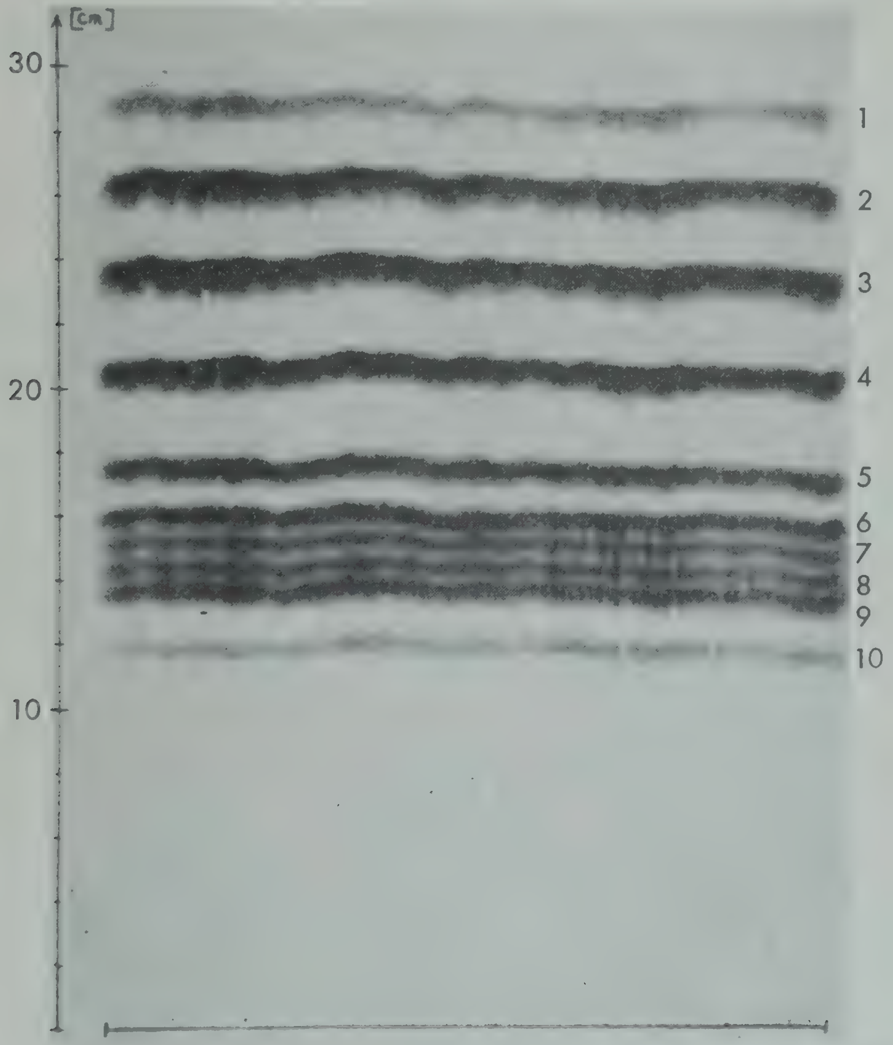


FIG. 4. Paper electrophoretic separation of amino acids at pH 2 (according to H. Bodemann). 1. Lysine; 2. Arginine; 3. Glycine; 4. Alanine; 5. Serine; 6. Leucine; 7. Methionine; 8. Glutamic acid; 9. Aspartic acid; 10. Tyrosine.

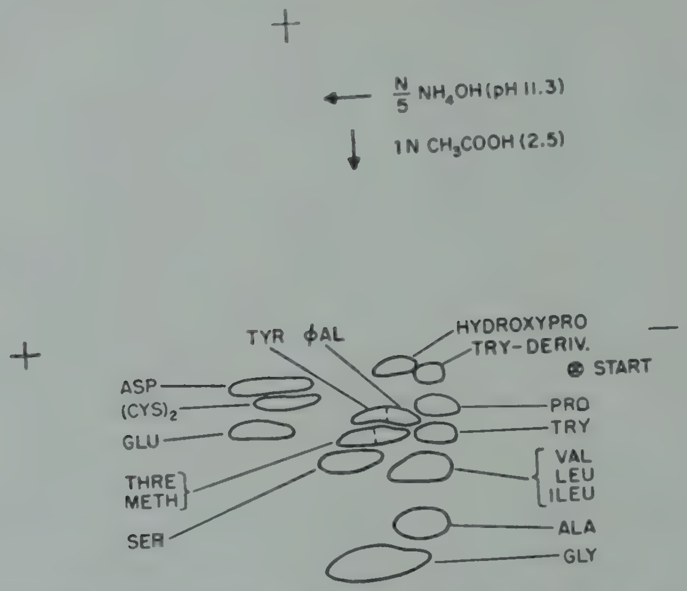


FIG. 5. Two-dimensional pattern of an amino acid mixture [according to Durrum (31)].

Another combined procedure employs paper electrophoresis in one direction, paper chromatography in the second, at right angles to the first (32). This method is particularly simple to use with the apparatus described under (22), on which sheets of paper 30 × 40 cm. may be used. Figure 6 shows such a "pherochromatogram" of 19 amino acids.

The separation of hydroxyproline and allohydroxyproline can be carried out with ease only by zone electrophoresis (pH 1.9). The allo compound travels 25% faster to the cathode (33). Several mixtures of amino acids have also been separated by continuous electrophoresis in an apparatus filled with glass powder or quartz sand (34). Excellent separations of amino acids in columns of cellulose powder have been reported by Porath (35).

1. Detection and Assay

The paper is sprayed with a solution of 0.2% ninhydrin in water-saturated butanol (36) or dipped into a 0.25% acetone solution of this reagent (37), then heated to 80–100°C. in a dry oven or left for several hours at room temperature to permit the development of the colored complexes. By spraying with Cu(II) salt (38, 39) the color hues of the various amino acid spots or bands can be transformed into a uniform stable dark red (40) and determined colorimetrically on the strip or after elution by comparison with standards. Polychrome dyeing with naphthoquinone-1,2-sulfonate (4, 41, 42) or isatin and ZnCl$_2$ was also employed (42). Upon heating to 100°C. (43) or after spraying with a solution of 4% xylose and 1.5% NaHSO$_3$ in 0.05 *M* phosphate buffer at pH 6.5–7

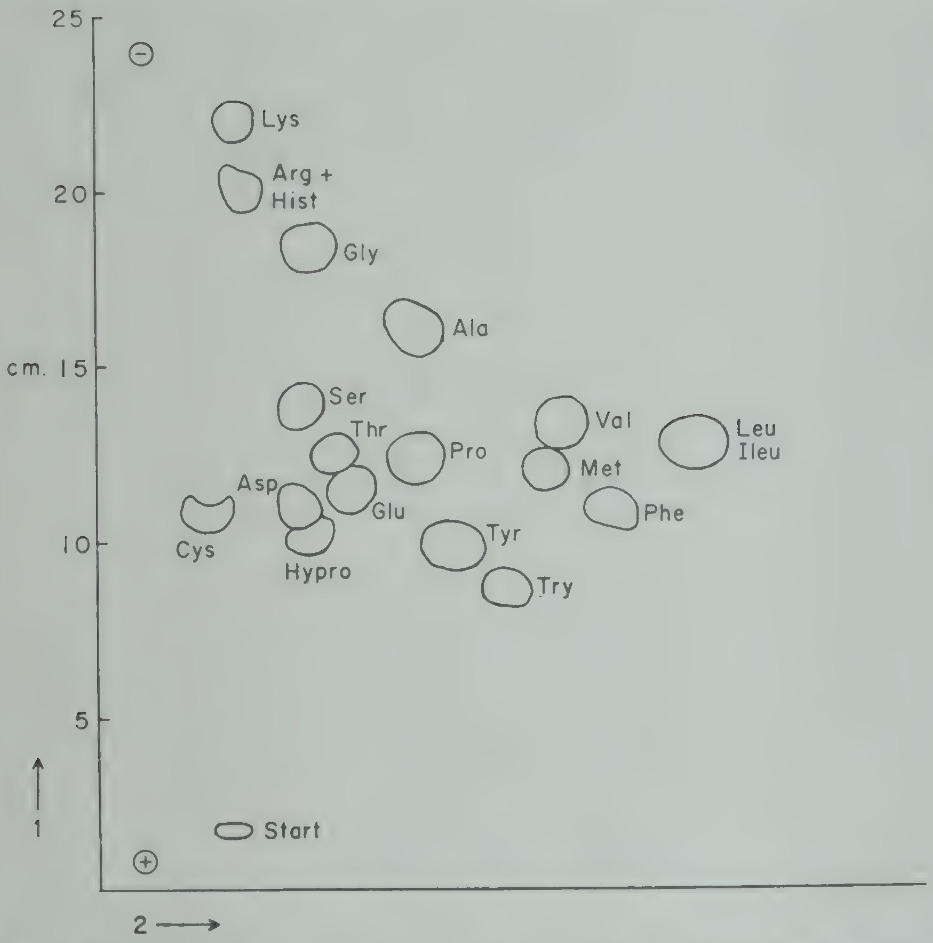


FIG. 6. Separation of nineteen amino acids on paper (according to P. Maslowski).
1 = Electrophoresis at pH 1.9, 60 volts per centimeter, 90 minutes.
2 = Ascending paper chromatography in *sec*-butanol-formic acid-water (75:15:10).
Abbreviations: Ala = alanine; Arg = arginine; Asp = aspartic acid; Cys = cystine; Glu = glutamic acid; Gly = glycine; Hist = histidine; Hypro = hydroxyproline; Ileu = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Phe = phenylalanine; Pro = proline; Ser = serine; Thr = threonine; Try = tryptophan; Tyr = tyrosine; Val = valine.

and heating to 80–90°C. for 1–1.5 hours, the amino acids yield spots which fluoresce in ultraviolet light (44). Particular sensitivity (limit of detection 1–1.5 μg cm.^{2}) is exhibited by the yellow fluorescence which appears in ultraviolet light after dipping into a 0.1% solution of *o*-cumaric acid in acetone and heating the air-dried sheets to 100°C. for 1–2 minutes (45). Furthermore, detection is possible by reaction with 2,4-dinitrofluorobenzene on the paper and bleaching of the yellow background with HCl gas. By this means, a quantitative spectrophotometric determination of the eluted yellow zones can be carried out (46). For the quantitative determination of amino acids and other Cu^{++}-binding substances on paper, see also "retention analysis" (47).

Specific detection reagents are: diazotized sulfanilic acid (Pauly reagent) for histidine and tyrosine; cinnamic aldehyde-HCl gas for tryptophan; *p*-dimethylaminobenzaldehyde in acid solution (Ehrlich reagent) for tryptophan; periodate followed by Nessler's reagent for vicinal aminohydroxy compounds; isatin for proline and hydroxyproline; phthaldialdehyde for glycine; β-napthol plus hypobromite (Sakaguchi reagent) for arginine; Na nitroprusside for cysteine and, after spraying with HCN, for cystine; and Folin's phosphomolybdate reagent for reducing amino acids.

As *derivatives of amino acids*, the *N*-dinitrophenyl amino acids can be separated by zone electrophoresis into four groups. At pH 1.5, dinitrophenylcysteic acid can be separated from all other DPN-amino acids (48). Guanido derivatives (49) are also separated.

B. Peptides

The first paper electrophoretic separation of (cyclo) peptides was carried out with α- and β-amanitin, substances found in the toxin of *Amanita phalloides* (50). Since then, the method has been applied successfully to numerous mixtures of peptides, for example for the separation of the liver or yeast peptides soluble in hot aqueous alcohol (51), or of the pituitary peptide hormones in starch (52, 53). A particularly instructive example of the usefulness of electrophoresis for the analysis of mixtures of peptides is provided by the separation of products of the partial degradation of insulin chains after oxidation of the SH groups to SO_3H groups (54). Figure 7 shows such an electrophoretic pattern on which thirteen different ninhydrin-positive components may be distinguished.

As for the amino acids, *detection* can be carried out here with the ninhydrin reaction which, however, becomes less intense with increasing chain length. A more sensitive method is the so-called "chlorine method." The pherogram is suspended for a short time in an atmosphere of chlorine, or dipped in a freshly prepared solution of chlorine in CCl_4, whereby the peptide linkages are transformed into —N(Cl)—CO—. After evaporation of the excess Cl_2 (and CCl_4), the chlorimine linkages may be made visible with KI-starch (55, 56) or benzidine (57) as blue spots. The removal of the excess chlorine from the paper is achieved in a few seconds by exposure to an atmosphere of NH_3 (58).

C. Amines

A detailed examination of the electrophoretic separability of amines on paper has been carried out by Weber (28), who was the first to recognize the advantage of strongly squeezed supports. After reduction of the normal absorbed water content of paper from about 200% to 100–150% by pressing it between two rotating rubber cylinders, a degree of moisture is achieved at which the interfering diffusion is greatly diminished. In order to protect the support against absorption of further fluid from the electrode vessels, cellophane membranes are interposed at the end which also reduce electroosmosis to a large extent. With these principles, the modern high-voltage electrophoresis apparatuses have been constructed (Section I, D). Table I lists the relative rates of migration of many amines at approximately 10 volts per centimeter, related to NH_4^+ = 1 in citrate buffer (10.5 gm. citric acid per liter, adjusted to pH 3.8 with NaOH) (28). The homologous primary alkylamines from C_1 to C_{10} are easily separated in 0.0075 *N* HCl (59). Other biologically important amines were determined quantitatively by Fischer and Bohn (60) with the ninhydrin-Cu method (38, 39) after paper electrophoretic separation (1, 22). Table II shows the

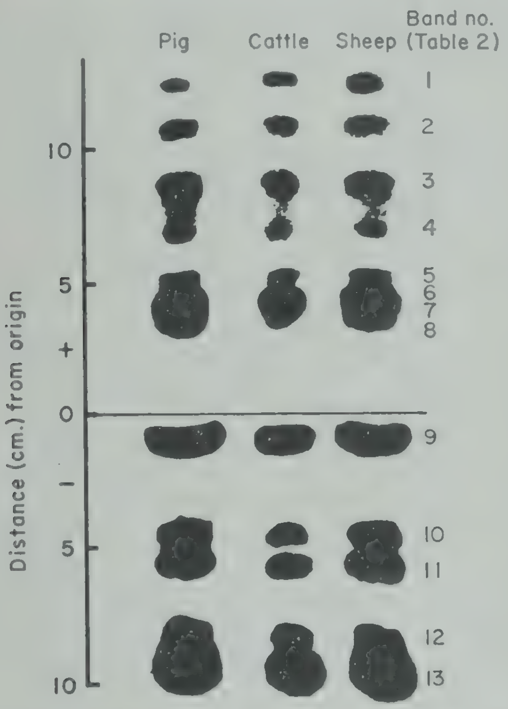


FIG. 7. Electrophoretic separations of peptic hydrolyzates of B fractions of cattle, pig, and sheep insulins. Pyridine-acetate buffer (pH 6.5); 28 volts per centimeter; 3 hours; colored with ninhydrin. [According to Sanger *et al.* (54).]

Amino acids found on hydrolysis: 1 = Glu, Val; 2 = Glu, Ala, Val; 3 = Glu, Ala, Val, Leu; 4 = Glu, Ala, Val, Leu; 5 = $CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg; 6 = $CySO_2H$, Glu, Gly, Val, Leu, Phe, Arg; 7 = $CySO_3H$, Glu, Gly, Tyr, Val, Leu, Phe, Arg; 8 = $CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg; 9 = All residues found in fraction 8; 10 = $CySO_3H$, Ser, Gly, Leu, His; 11 = $CySO_2H$, Asp, Glu, Ser, Gly, Val, Leu, Phe, His; 12 = Thr, Ala, Tyr, Pro, Lys (Phe); 13 = Thr, Ala, Tyr, Pro, Lys.

TABLE I

RELATIVE RATE OF TRAVEL OF AMINES[a]

Substance	Rate of travel
Urea	0
Glycine	0.04
Mescaline	0.24
Arginine	0.25
Histidine	0.26
Lysine	0.26
Gramine	0.27
Ornithine	0.28
Tyramine	0.29
Ephedrine	0.32
Creatine	0.37
Phenylethylamine	0.41
Histamine	0.58
Propylamine	0.61
Cadaverine	0.64
Putrescine	0.67
Ethylamine	0.72
Dimethylamine	0.81
Methylamine	0.92
NH_4^+, K^+	1.0

[a] Compared with the speed of K^+ and $NH_4^+ = 1$.

travel distances in centimeters after 2 hours at 0°C. and 15 volts per centimeter in various buffer solutions, each of which is suitable for the clean separation of different groups.

Glucosamine, and probably other *amino sugars* as well, can be separated in veronal buffer at pH 8.6 from neutral amino acids and the more rapidly migrating hexonic bases (61).

D. Sugars and Polyalcohols

1. Mono- and Disaccharides

a. Electrophoresis with borate. Sugars and polyalcohols, being nonpolar molecules, do not migrate in the electrical field. But they form anionic complexes with boric acid (62), so that migration to the anode takes place when borate buffers are the electrolyte (20, 63, 64). Consden and Stanier (64) measured the dependence of the mobility (cm^2/volt-second) on the pH of the 0.05 *M* borate buffer for eight monosaccharides and two disaccharides (about 10 volts per centimeter, 3–4 hours) on filter paper and

TABLE II

TRAVEL DISTANCES[a] OF AMINES IN VARIOUS BUFFER SOLUTIONS

Amine	Buffer A	Buffer B	Buffer C
Spermine	− 7.8	−12.0	−10.2
Spermidine	− 9.6	−13.0	−13.0
Histamine	−10.8	−13.0	−15.2
Colamine	−10.6	−10.5	−16.2
Cadaverine	−11.8	−13.0	−17.4
Putrescine	−12.7	−14.0	−18.2
Trimethylenediamine	−13.2	−15.4	−18.4
Lysine	− 5.1	− 5.5	− 8.0
Buffer solutions:	A	B	C
Pyridine	40 cc.	50 cc.	40 cc.
Glacial acetic acid	30 cc.	50 cc.	20 cc.
Citric acid	12 gm.	—	20 gm.
Water	930 cc.	900 cc.	940 cc.

[a] Distance in centimeters after 2 hours at 0°C. and 15 volts per centimeter.

obtained the values listed in Table III. It may be seen that all the sugars exhibit an increasing mobility with rising pH, though not to the same degree. This permits the selection of pH ranges in which certain monosaccharides can be separated effectively. The increase in the rates of

TABLE III

MOBILITIES OF SUGARS[a] AT 20°C. IN BORATE AT VARIOUS pH VALUES ON WHATMAN NO. 1 PAPER[b]

Sugar	pH 7.0	pH 8.0	pH 8.6	pH 9.2	pH 9.7
Fructose	8.2	9.7	11.4	12.5	13.1
Sorbose	8.7	10.4	12.2	14.1	14.3
Glucose	2.4	6.5	11.4	14.5	14.6
Galactose	2.8	5.8	9.6	13.0	13.1
Mannose	2.6	4.9	7.8	9.8	10.0
Ribose	7.0	9.1	10.2	10.9	11.0
Arabinose	3.2	6.5	10.3	13.3	13.9
Rhamnose	1.3	2.4	4.4	7.1	7.8
Cellobiose	0.5	0.5	1.5	3.2	4.5
Raffinose	0.5	0.9	1.7	3.6	4.8

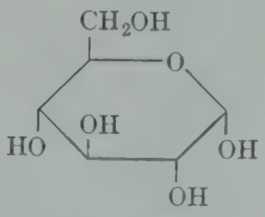
[a] cm^2/volt-second × 10^5.

[b] Consden and Stanier (64).

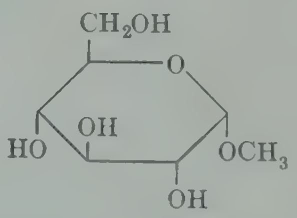
migration with pH most probably may be traced in part to the increased dissociation of the acid borate complexes. The differences in mobility are caused by the different number of complexed boric acid molecules and in the different constants of complex formation, which again depend on the structure.

A comparison of the rates of migration of single monosaccharides with those in which one or more OH groups are blocked systematically by methylation provides an insight into the structural prerequisites for borate complex formation. This problem has been examined by Foster and collaborators in a series of informative investigations. Foster measured the migration of the sugars in 0.2 *M* borate buffer at pH 10 on Whatman No. 3 paper relative to glucose; the results are expressed as the M_G value (65).

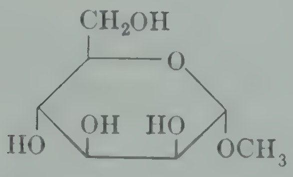
The low M_G value of 0.11 for α-methyl-D-glucopyranoside (66) indicates, for instance, that the well-known preferential reaction of *cis*-placed hydroxyles with borate plays a principal role in complex formation (I).



α-D-Glucopyranose
$M_G = 1$



α-Methyl-D-glucopyranoside
$M_G = 0.11$



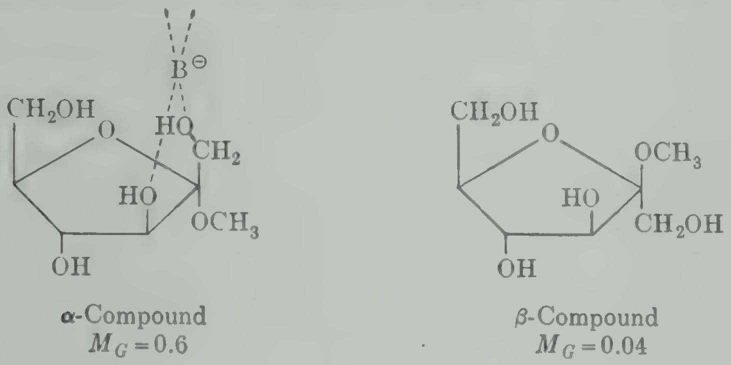
α-Methyl-D-mannopyranoside
$M_G = 0.42$

(I)

α-Methyl-D-mannopyranoside with two *cis*-placed OH groups has shown a higher M_G value of 0.42, which, however, is still far below that of glucose. The latter, therefore, does not appear to exist exclusively as glucopyranose with only two *cis*-placed OH groups. Comparisons with several systematically changed derivatives, in which one or more defined OH groups are blocked, lead to the conclusion that glucose reacts with borate at pH 10 with all its equilibrium forms:

aldehyde ⇄ α- and β-pyranose ⇄ α- and β-furanose so that all its OH groups are able to participate in complex formation (67).

That not only *cis*-placed hydroxyls of 1,2-glycol take part in the complex formation may be deduced, among other things, from the fact that α-methyl-D-fructofuranoside has a high M_G value of 0.60, while the diastereomeric β-compound has a value of only 0.04 (formula II).



(II)

This indicates that *cis*-placed OH and —CH₂OH— groups also make possible the formation of a (6-membered) borate complex (68). Numerous M_G values of monosaccharides and their methylated derivatives may be found in the original publications (65–69). Table IV, on the other hand, provides a summary of the M_G values of several disaccharides. The use of higher voltages (40–60 volts per centimeter) leads to particularly sharp separations in this instance as well (30).

TABLE IV

M_G VALUES OF SEVERAL DISACCHARIDES

Disaccharide	M_G value
Melibiose	0.80
Gentiobiose	0.75
Isomaltose	0.69
Laminariabiose	0.69
Lactose	0.38
Maltose	0.32
Cellobiose	0.23

The electrophoretic separation of sugars in borate buffer has been carried out successfully also on *glass-fiber paper* (5, 70). The separability (M_G value) is about the same as on paper, but all the substances travel at a greater absolute rate. An advantage is the chemical stability of this support in contact with corrosive detection reagents (cf. below), which react in a disturbing manner with paper.

b. Electrophoresis without borate. Reaction with benzylamine transforms sugars into benzylglycosamines which may be separated as cations in acidic buffers (71). Sugars and polyalcohols may also be caused to travel at different rates in various other electrolyte solutions without direct chemical treatment. Frahn and Mills (72) have studied this in detail and have found, for example, that, in a 1% solution of *arsenite* at pH 9.6 on paper migration to the anode takes place with the following values: glucose = 1, mannose = 2.4, fructose = 5.1. A reaction also takes place in a solution of *lead* acetate; it consists of the formation of *cationic* complexes which cause the sugars under test to migrate at different rates to the cathode: glucose = −0.7, mannose = −4.5; fructose = −2.8.

Since reducing sugars also react with *hydrogen sulfite* with the formation of an α-hydroxysulfonate, they may also be caused to travel to the anode on a paper strip which is saturated with a 0.4 *M* solution of $NaHSO_4$ (73). But the rate of the addition reaction is so slow (and different for different sugars) that during electrophoresis only a portion of the applied amount of material travels to the anode, remaining connected to the nonreacted portion by a trail. The distance between the two spots, a measure of the true mobility of the addition product, can be used for estimation of the *molecular weight* of the sugar being studied. For example, related to glucose = 1, it is 1.13–1.16 for pentoses, 0.70 for disaccharides, 0.55 for maltotriose and panose, and 0.45 for maltotetraose. In 0.1 *M* buffered hydrogen sulfite solution at pH 4.7, migration of reducing sugars also takes place, and by working at 60°C. one encounters them only as bisulfite compounds, that is, as uniform spots (74). Since other aldehydes and ketones react much more rapidly with hydrogen sulfite even at room temperature, this electrolyte represents a valuable medium for their paper electrophoretic separation (cf. Section II, E).

2. Polyalcohols

For this class of compounds, the same considerations concerning the ability to form complexes hold as for the carbohydrates, of course with the exception of the reaction with hydrogen sulfite. Michl (20) achieved a separation of glycerol and mannitol on paper in a solution of 0.08 *M* borax. Later Gross (75) carried out further successful investigations on meso-erythritol, sorbitol, mannitol, and dulcitol, among others. The different electrophoretic behavior of hexitols in arsenite and lead acetate on paper, as well as an indication of their rates of migration in borate solution at pH 9.2 (cf. Table V), was described in the paper of Frahn and Mills (72) cited above.

The cyclitols have been subjected to fairly detailed investigation. The various isomeric inositols provide a beautiful illustration of the dependence

TABLE V

MIGRATION OF SOME HEXITOLS[a]

Hexitol	In borate	In arsenite	In lead acetate
Mannitol	+14.1	+7.5	−2.8
Dulcitol	+15.7	+6.3	−3.6
Sorbitol	+12.8	+9.3	−4.6

[a] Centimeters after 2 hours; 17 volts per centimeter.

of the rate of migration in borate (given here as the M_G value) on the relative position of the hydroxyl groups (76), since the mobility increases approximately with the number of the neighboring *cis*-placed OH groups (Table VI). The special position of mucoinositol requires an additional explanation. For further investigations, see (77).

TABLE VI

RATE OF MIGRATION IN BORATE OF VARIOUS ISOMERIC INOSITOLS

Compound	M_G value	*cis*-OH groups
Scyllo-inositol	0.05	None
Meso-inositol	0.51	1:2:3
(+) or (−) inositol	0.63	1:2, 5:6
Epi-inositol	0.73	1:2:3:4:5
Allo-inositol	0.88	1:2:3:4:5:6
Muco-inositol	0.96	1:2, 4:5

Detection of carbohydrates and polyols. Reducing sugars can be visualized with aniline-phthalic acid, ammoniacal silver nitrate, triphenyltetrazolium chloride, or naphthoresorcinol, among other reagents. For polyols in general, a reaction with periodate-permanganate has been proposed. The aldehydes produced by the action of periodate are oxidized by MnO_4^-, and the resulting MnO_2 is reacted with benzidine, yielding a sensitive blue color (78). In this reaction as well as in that with alkaline permanganate solution which is reduced by polyols, paper as a support interferes because of its structural similarity (polysaccharide). In such instances, the use of glass-fiber paper as support is to be recommended (5). For quantitative evaluation, it is best to elute the pherogram and to determine the concentration in the eluates by standard methods of sugar determination. For the removal of borate, which is eluted too and which may interfere under certain circumstances, the dried residue from the eluate is treated with 1% methanolic HCl and the boric acid trimethyl ester is evaporated *in vacuo* (79).

Electrophoresis in borate is also successful with natural glycosides. Some applications are illustrated under *nucleosides* (Section II, F, 2) and flavonol glycosides (Section II, J, 2).

E. Aldehydes and Ketones

The possibility described in the previous section of allowing sugars to migrate electrophoretically as bisulfite compounds on $NaHSO_3$-impregnated paper (73) has been elaborated by Theander (74) for a study of aldehydes and a few ketones. This class of compounds, for which there is no other simple electrophoretic method, reacts rapidly with HSO_3^-, so that the compounds which have been studied migrate uniformly toward the anode as α-hydroxysulfonates in 0.01 or 0.1 M bisulfite solution. A sampling of M_v values (migration relative to vanillin = 1) in 0.1 M buffer is given in Table VII. Detection of the separated compounds takes place by heating with anisidine-HCl or silver nitrate-$NaOC_2H_5$ for reducing and with dinitrophenylhydrazine-HCl for all carbonyl compounds.

TABLE VII

RATE OF MIGRATION[a] OF VARIOUS ALDEHYDES AND KETONES

Substance	M_v value	Substance	M_v value
n-Heptanal	0.98	Benzaldehyde	1.16
Diacetyl	0.69	Salicylaldehyde	0.95
Citral	1.36	*m*-Hydroxybenzaldehyde	1.16
Cinnamic aldehyde	1.18	Veratric aldehyde	1.03
Cyclohexanone	1.14	Protocatechualdehyde	1.00
Cyclohexan-1,3-dione	1.78	Orthovanillin	1.10
Furfural	1.29	Vanillin	1.00

[a] Relative to vanillin = 1 (M_v value).

By reaction with benzsulfhydrotamic acid, only aldehydes are converted to hydrotamates, which can be separated by paper electrophoresis (79a) and detected after spraying with Fe^{3+}-salts.

F. Building Blocks of Nucleic Acids

1. Nucleic Acid Bases

The complete electrophoretic separation of the *bases of nucleic acids* is relatively difficult to achieve. At pH 3–3.5, the most basic compound, adenine, migrates to the cathode while the others (thymine, uracil, cytosine) remain in one spot (80). In borate (pH 10) the purines and pyrimidines (81) travel as follows (M_G values; cf. page 000): thymine 0.42–0.59 (with tailing); uracil, 0.95; cytosine, 0.0; and adenine 0.35–0.56 (with tailing

2. *Nucleosides*

The use of borate buffer has been of greater advantage with nucleosides (82). The ribonucleosides, deoxyribonucleosides (Table VIII), as well as numerous synthetic pyranosidonucleosides can be separated clearly on paper at pH 10.

TABLE VIII

M_G VALUES OF SOME NUCLEOSIDES IN BORATE BUFFER[a,b]

Ribonucleosides	M_G	Deoxyribonucleosides	M_G
Uridine	1.05	Thymidine	0.51
Cytidine	0.64	Deoxycytidine	0.0
Adenosine	0.60	Deoxyuridine	0.67
Guanosine	0.95	Deoxyadenosine	0.06
		Deoxyguanosine	0.59

[a] From Burke (81).

[b] 0.2 M, pH 10.

3. *Nucleotides*

The nucleotides are most easily separated by paper electrophoresis. For example, in 0.05 M acetate-acetic acid at pH 2–8, the sequence of migration is uridylic acid (fastest), guanylic acid, adenylic acid, cytidylic acid (83). In a series of investigations, Markham and Smith have been able with good success to separate not only mono- but also di- and trinucleotides and 2′, 3′-cyclophosphates at higher voltages under cooling in acetate or phosphate buffer at pH about 7 (26, 83a). In this way, the migration values of 10 of the 16 possible ribonucleotides in 0.05 M ammonium formate at pH 3.5 have been determined and all were found to differ from each other; many trinucleotides could also be separated from each other. Mention should finally be made in this area of the possibility of paper electrophoretic separation of isomeric nucleotides which differ by the position of the esterified phosphoric acid in the 2′, 3′, or 5′ position (23). Figure 8 shows such separations in various buffer solutions. The separation of nucleotides on a rayon thread (18) has already been mentioned on page 496.

The detection of the separated substances for bases, nucleosides, and nucleotides is best accomplished by quenching of the fluorescence of the paper or of fluorescent compounds applied to it (84) in ultraviolet light or by contact copying in ultraviolet light on light-sensitive paper which is less

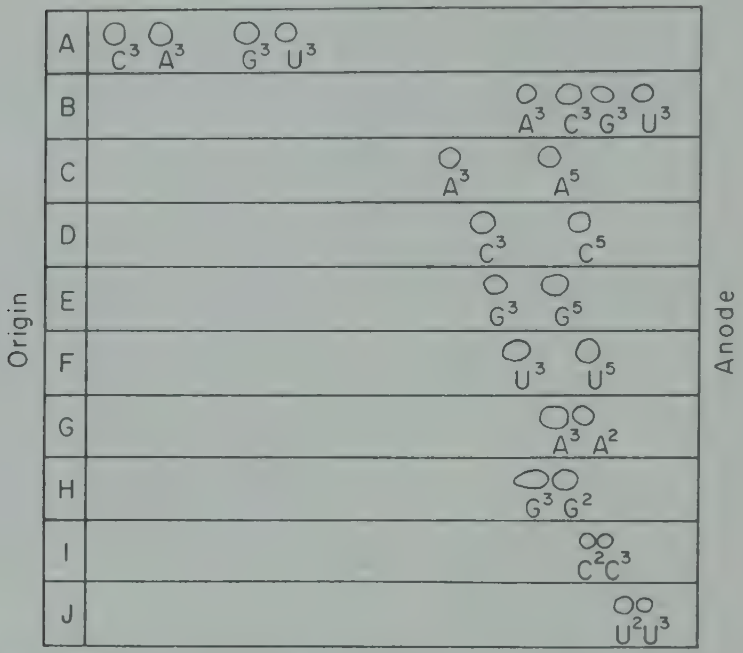


FIG. 8. Resolutions of nucleotides [according to Crestfield and Allen (23)].

A, *G*, *C*, and *U* refer to adenylic, guanylic, cytidylic, and uridylic acids, respectively. Superscript refers to appropriate isomer. Length of paper shown is 28 cm.

A. Formate buffer, pH 3.5, ionic strength 0.1, field 30 volts per cm., time 60 minutes.
B. 0.1*M* sodium tetraborate solution, pH 9.2, field 30 volts per cm., time 93 minutes.
C–F. 0.1*M* sodium tetraborate solution, pH 9.2, field 37 volts per cm., time 90 minutes.
G. 0.1*M* sodium bicarbonate, pH 8.0 field 40 volts per cm., time 120 minutes.
H. Formate buffer, pH 3.8, ionic strength 0.1, field 38 volts per cm., time 240 minutes.
I. Phosphate buffer, pH 6.2, ionic strength 0.1, field 32 volts per cm., time 125 minutes.
J. Phosphate buffer, pH 5.8, ionic strength 0.1, field 37 volts per cm., time 148 minutes.

strongly blackened at the absorbing spots (photoprint) (26). For quantitative determination it is best to use the measurement of extinction at the absorption maximum (260 mμ). Concerning the detection of phosphoric acid, also in these derivatives, see Section II, H.

4. Nucleotide-like Coenzymes

The phosphate-transferring system of the adenine-5′-nucleotides, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), can also be separated by paper electrophoresis. This analysis is often difficult to reproduce and works best at about pH 3. Wade and Morgan (85) use a 4.6% butyric acid-NaOH buffer and Whatman paper which is washed free of copper and magnesium with 1 *N* formic acid. Recently, Cerletti *et al.* (86) reported successful separations in 0.05 *M* ammonium formate at a pH adjusted very precisely to 3.02. Then the compounds indicated in Fig. 9 also separate in a Flodin

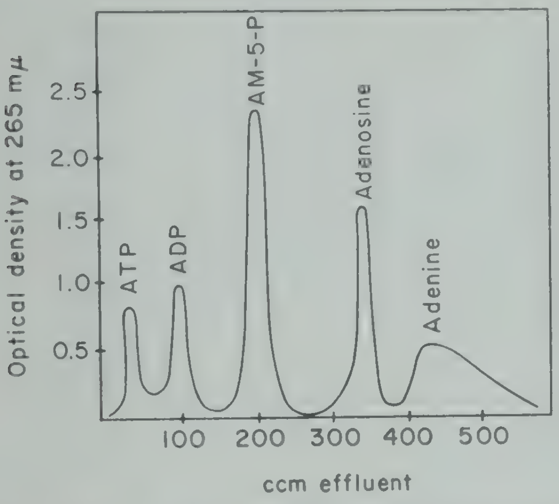


FIG. 9. Separation by column electrophoresis of a mixture containing 1.5 mg. each of ATP, ADP, adenosine, and adenine, and 2 mg. AM-5-P. Whatman cellulose powder; ammonium formate buffer pH 3.02, 0.05 *M*, 300 volts; 14 milliamperes for 18 hours.

column (83) on cellulose powder with the sharpness shown by the measurement of absorption (at 265 mμ in the fractionally collected eluate).

Flavine adenine dinucleotide (FAD) and flavine adenine mononucleotide (FMN) can be separated well from each other and from riboflavine on paper in ammonium acetate at pH 6.8 in the manner illustrated in Fig. 10, A (87). In the same paper, there is also a description of the nucleotide separation shown in Fig. 10, B, which indicates, among other data, the excellent separation of triphosphopyridine nucleotide (TPN) from diphosphopyridine nucleotide (DPN).

Coenzyme A and the intermediates in its biological synthesis in microorganisms have also been analyzed by paper electrophoresis after tagging with β-alanine-C^{14} (88).

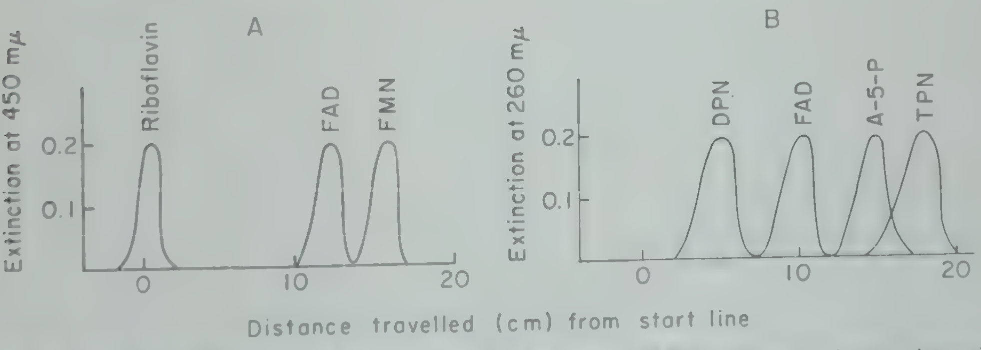


FIG. 10. (A) Separation of riboflavine, FAD, and FMN by paper electrophoresis. Ammonium acetate pH 6.8, 5 hours, 600 volts, 6 milliamperes. (B) Separation of FAD, DPN, TPN, A-5-P by paper electrophoresis. Ammonium acetate pH 6.7, 4½ hours, 600 volts, 10 milliamperes.

G. Nucleic Acids, Polysaccharides, and Other High Polymers

1. Nucleic Acids

Ribonucleic acids (RNA) and deoxyribonucleic acids (DNA) can be separated in veronal buffer at pH 8.6 or phosphate buffer at pH 8.0 at 5–10 volts per centimeter by paper electrophoresis of several hours' duration, wherein DNA travels more rapidly to the anode. Visualization is carried out with *methyl green-pyronine* according to Unna. RNA is colored red, DNA bluish-green in this reaction (89). Micropreparative separations in the same manner have been carried out for these two substances in the continuous set-up on paper (90), also in 0.01 *M* citrate at pH 5.0 (91). In the acid range (pH 2–3) a decomposition of both nucleic acids into three components each has been observed during paper electrophoresis (92, 93).

2. Polysaccharides

The electrophoretic separability of glycogen and galactogen was demonstrated for the first time in 1953 by Geldmacher-Mallinckrodt and associates (6, 94). These authors used veronal buffer at pH 9 and *silk fabrics* as supports, since the detection of the substances with the Molisch reagent (α-naphthol-H_2SO_4) after hydrolysis with 2 *N* HCl at 115°C. was impossible because of their chemical similarity to paper. Later, Fuller and Northcote (95) employed 0.1 *M* borate buffer at pH 9.3, working under toluene cooling at 50 volts per centimeter (90 minutes) on paper, silk, or

TABLE IX

RATIO OF MOVEMENT OF POLYSACCHARIDE TO MOVEMENT OF YEAST MANNAN

Substance	Paper	Silk	Glass-fiber paper[a]
Yeast mannan	1.00	1.00	1.00
Yeast glycogen	0.49	0.58	0.45
Snail galactan	1.11	1.33	1.25
Elecampane inulin	0.25	0.25	0.28
Amylose	Origin	Origin	0.38
Amylopectin	0.27	0.42	0.45
Wheat straw xylan	—	Origin	0.45
Apple pectin	—	—	1.53
Dextran A	—	0.33	0.33
Dextran B	—	0.50	0.33

[a] Reeve Angel and Co. Ltd., London

glass-fiber paper. They obtained fairly good separations of the materials traveling to the anode; these are summarized in Table IX.

By means of electrophoresis in a column filled with homogeneous glass powder (cf. page 495), polysaccharides may be obtained *preparatively* in 0.05 *M* borate buffer at pH 9.2 (9). In this manner, mixtures of glycogen and mannan or of inulin, glycogen, mannan, and galactan in amounts of 10 mg. each could be separated satisfactorily.

Electrophoresis of mucopolysaccharides has been carried out with Hyflo-Super Cel as support (96, 97).

3. Other High Polymers

In this field, very few experiments have been described until now. *Polyacrylic acid* migrates well in the buffers used for protein electrophoresis and may be detected in aqueous-alcoholic solution by dyeing with carbol fuchsin (98). Polyvinylpyrrolidone also migrates and is colored with Lugol solution (99).

Finally, it should be mentioned here that humic acids and similar high polymers which are obtained from hydroquinone by alkaline oxidation, can be separated electrophoretically (100).

H. Phosphoric Acids and Esters

Orthophosphate, pyrophosphate, and triphosphate may be separated in the stated order by paper electrophoresis at pH 10 (Na borate-NaOH),

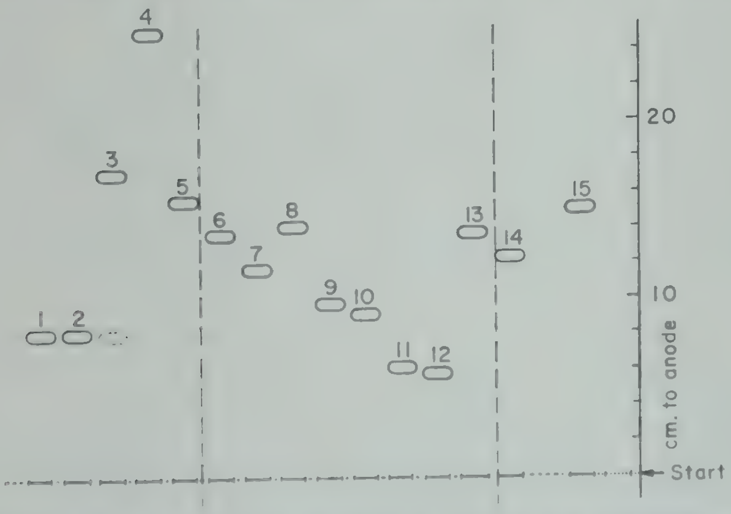


FIG. 11. High-voltage separation of various phosphorus compounds at pH 1.9: 50 volts per centimeter, 90 minutes. *1.* H_3PO_4; *2.* H_3PO_3S; *3.* $H_4P_2O_7$; *4.* $H_3(PO_3)_3$; *5.* H_3PO_3; *6.* methyl phosphate; *7.* ethyl phosphate; *8.* diethyl phosphate; *9.* *n*-butyl phosphate; *10.* *sec*-butyl phosphate; *11.* *tert*-butyl phosphate; *12.* octyl phosphate; *13.* phenyl phosphate; *14.* *S*-*n*-butyl thiophosphate; *15.* acetyl phosphate. (From Wieland and Lambert (102).)

though with difficulty; PO_4^{---} migrates most rapidly. In the same system, one can also separate the mixtures of trimetaphosphate (most rapid), triphosphate and Graham salt [$Na(PO_3)_x$], and other more highly condensed phosphates (101).

In the acid range, the opposite behavior is observed (102). As shown in Fig. 11, left portion, trimetaphosphate migrates farthest at pH 1.9 and pyrophosphate migrates twice as far as orthophosphate. Phosphorous acid, by its faster migration, proves to be a stronger acid than orthophosphoric acid; thiophosphoric acid proves to be about as strong. Among the phosphoric acid esters shown in Fig. 11, center portion, large differences can also be observed which are derived from the clear-cut difference in dissociation constants. The following are the pK_1 values (103):

Monomethyl phosphate	1.54
Monoethyl phosphate	1.60
Mono-*n*-butyl phosphate	1.89
Diethyl phosphate	1.39

It can be seen that the migration of these esters occurs in exactly the way which may be deduced from these values.

The biologically important fructose phosphates can also be separated on paper in veronal buffer at pH 8.6, since under these conditions fructose-1,6-diphosphate migrates more rapidly than fructose-6-phosphate (104). At pH 6.5 in pyridine-acetic acid-water with high voltage, one may also separate the isomers dihydroxyacetone phosphate (faster) and phosphoglyceraldehyde (105).

Detection. Phosphoric acid and its derivatives are visualized by spraying with an ammonium molybdate solution containing $HClO_4$ or HCl. By heating the sprayed sheets, the derivatives are hydrolyzed and then reduced with H_2S to molybdenum blue (106). Reduction to the dye takes place even under irradiation with ultraviolet light (107).

Concerning paper electrophoresis of various alkylphosphonic acids, see (108).

I. Organic Acids and Phenols

1. Organic Acids

In the series of *aliphatic fatty acids*, the results of an attempt at electrophoretic separation on a cotton wick of 86-cm. length with 3 *N* aqueous ammonia have been reported (109). Stearic, palmitic, myristic, and lauric acids were separated in 36 hours at 300 volts by their increasing rate of migration in the stated order. Later, this analysis was also carried out on filter paper (110). It is assumed that not only the small differences in molecular weight but primarily adsorption effects are responsible for the presence of these differences in migration. It has been possible also to separate all the fatty acids on paper in 0.2 *N* glycerol in NaOH at 90°C. (111). Detection is carried out with indicators. At 100 volts per centimeter in 0.1 *M* ammonium carbonate solution, pH 8.9, the separation of the lower fatty acids (C_1 C_{10}) proceeds very well on paper in less than 1 hour.

With α-keto acids, a paper electrophoretic separation of the 2,4-dinitrophenylhydrazones of pyruvic, oxalacetic, and α-ketoglutaric acids in 0.05 *M* Na_2CO_3 solution has been described (112). Berbalk and Schier (113) have demonstrated the expected dependence of the mobilities on the dissociation constants *K* in a paper electropherogram (0.5 *N* formic acid, pH 2, at 10°C.) for a considerable series of organic acids including *hydroxy* and *dicarboxylic acids*. Table X summarizes the results. Particularly sharp separations are also achieved in the series of organic acids by the use of higher voltage (20, 114).

TABLE X

Dependence of Mobilities of Organic Acids on the Dissociation of Constants (*K*) in Paper Electrophoresis

Acid	$K \times 10^4$	$\mu \times 10^5$
Oxalic acid	650	9
Dichloroacetic acid	330	6.5
Maleic acid	120	6.7
Pyruvic acid	30	3.7
Monochloroacetic acid	15	2.9
Aconitic acid	15	2.4
Fumaric acid	10	2.2
Tartaric acid	9.8	2.3
Citric acid	8.3	1.9
Malic acid	3.8	1.4
Glyceric acid	2.3	1.2
Itaconic acid	1.5	0.7
Lactic acid	1.4	0.7
Succinic acid	0.64	0.2
Glutaric acid	0.47	0.1

It is also possible to separate the otherwise only difficultly separable isomeric *β-naphthol-1-* and *β-naphthol-3-carboxylic* acids in pyridine-glacial acetic acid-water (pH 6) in a high-voltage apparatus under cooling (115).

Of course, *sulfonic* acids may also be separated electrophoretically if their molecular weights are clearly different from each other. This holds, for instance, for the low-molecular condensation products of formaldehyde and *p*-phenolsulfonic acid which were separated in a horizontal tube filled with *agar gel* and evaluated by the direct measurement of absorption in transmitted ultraviolet light (116).

2. Phenols

For phenols it is also true that their distance of electrophoretic migration to the anode is directly proportional to their dissociation constant. This was demonstrated, for example, in the experiments with picric acid and 2,4-dinitrophenol cited under (113) in which only the former exhibits appreciable mobility at pH 2. Concerning the separation of isomeric mono- and dinitro-phenols on paper, cf. (117).

With phenols containing several OH groups, the rate of migration may be influenced further by *borate* buffer, since it forms very strong complexes with those having *ortho*-placed hydroxyls. Thus, gallic acid ester and pyrogallol in 0.11 M borate solution, to which a small amount of hydrogen sulfite is added to prevent the air oxidation of the phenols, are easily separated by their anodic migration from the monophenols and other diphenols (20). Detection is carried out here with $FeCl_3$ solution. The use of borate buffer is also recommended for the separation of the nitration products of diphenols (118).

J. Dyes

1. Synthetic Dyes

The electrophoretic separability of a dye mixture (e.g. methyl red and methyl orange (119)) was first demonstrated by Strain in 1939 with a chromatographic column of Hyflo-Super Cel or glass beads, and the term "electrochromatography," proposed also for other zone electrophoreses, was coined for this process. Later, numerous dye mixtures were separated electrophoretically, in particular on paper, of which only the continuous separation of acid fuchsin into several bands (120) and that of several acridine dyes in 0.1 N HCl as electrolyte (121) will be mentioned here. In the latter example, quinacrine migrates most rapidly, followed by 5-aminoacridine, acriflavine, and proflavine. For dyes which are only slightly soluble in water, one may use even *organic solvents* such as nitromethane-acetic acid or absolute alcohol (122). This possibility extends the scope of application of zone electrophoresis into a general analytical tool of preparative organic chemistry.

2. Natural Dyes

For natural dyes, insofar as they are of acidic, basic, or phenolic nature, the principles described for these classes of substances apply. For instance, *porphyrins* are easily separated, on the basis of the number of carboxyl groups they carry, in agar gel containing $M/15$ phosphate buffer at pH 8. Even the isomeric coproporphyrins I and III could be separated, although a tube of 80-cm. length had to be used (123). Concerning paper electrophoretic separation of porphyrin pigments, see (124).

A beautiful example of preparative application is provided by the production of crystallized *betanin*, the bright red-violet dye of the red beet. It was isolated by electrophoresis of an enriched product on a 1-meter long paper powder column in pyridine-formic acid buffer at pH 4.5 (125). The crude juice of the beet shows red and yellow components which travel to the anode at different rates in a paper pherogram (0.1 M citrate buffer at pH 5.5) (126).

For natural dyes which contain several OH groups, borate is a suitable electrolyte which forms complexes with *o*-diphenol groups as well as with *cis*-placed OH groups of glycosidically bound sugars. Ten flavonol glycosides (127) studied in 2% borate solution on paper migrate to the anode according to these criteria in the following sequence of increasing rate: robinin-morin, acacetin-hesperidin-maringin-lutidine-7-glucoside, myricetin, quercetin, rutin, myricitrin.

K. Biologically Active Substances

This very heterogeneous section contains a sampling of the applications of electrophoresis to some natural materials.

1. Vitamins

The derivatives of vitamin B_6 may be separated on paper in acetate buffer at pH 5.1 according to this scheme (128):

⊖ pyridoxamine, pyridoxine, pyridoxal, pyridoxamine phosphate (starting point), pyridoxal phosphate ⊕.

This publication (128) also provides data on the electrophoretic behavior of derivatives of vitamin B_2.

The presence in feces of some basic compounds with microbiological vitamin B_{12} activity, in addition to the neutral vitamin, was demonstrated by paper electrophoresis (2 N acetic acid, HCN atmosphere) (129).

2. Hormones

The pituitary hormones have been mentioned among the peptides (cf. page 506); at this point, only the *steroids* will be discussed briefly. Since they are electrically neutral and difficultly soluble in water, those steroids that contain OH groups are transformed according to Voigt and Beckmann (130) into the anodically migrating half-esters of succinic acid by reaction with succinic acid anhydride. The esters, then, can be separated in borate buffer (pH 8.25) and detected, for example, by their fluorescence after spraying and heating with 15% phosphoric acid or 10% sodium hydroxide. The sequence of several steroid hormones is shown in the scheme (131):

↓ ——— starting point
Testosterone
⊕ Cortisone
Deoxycorticosterone
17-Hydroxycorticosterone

The natural steroid sulfates and glucuronates of urine may also be separated by paper electrophoresis in veronal buffer at pH 8.6 containing 20% ethanol (132).

3. *Antibiotics*

To give an example, we may describe the application of paper electrophoresis to the search for antibiotic substances (133). The culture fluids of the microorganisms were studied in comparison to chloromycetin by paper electrophoresis in buffers of different pH values and any antibiotics present were documented by bioautography. The paths of migration permit conclusions concerning the basic or acidic nature and isoelectric behavior of the materials.

4. *Alkaloids*

Scopolamine and hyoscyamine were separated by Deckers and Schreiber (134) by paper electrophoresis in $N/15$ borate buffer at pH 8.6, and brucine and strychnine were separated in citrate buffer at pH 3.5. The electro-

TABLE XI

ELECTROPHORETIC MIGRATION OF ALKALOIDS IN 5 N ACETIC ACID[a,b]

Alkaloid	Migration (mm.)	Alkaloid	Migration (mm.)
Aconitine	32	Hyoscyamine	46
Atropine	45	Lobeline	46
Brucine	40	Nicotine	99
Caffeine	5	Quinine	74
Cinchonine	80	Solanine	34
Coniine	105	Strychnine	48
Ephedrine	52	Sparteine	76

[a] From Kariyone *et al.* (135).

[b] 750 volts, 0.5 milliamperes per centimeter, 2 hours.

phoretic migration of a large number of alkaloids in 5 *N* acetic acid (750 volts, 0.5 milliamperes per centimeter, 2 hours) was measured by Kariyone *et al.* (135). A sampling of the results is presented in Table XI. The detection of the bases is usually accomplished by spraying with a modified Dragendorff reagent (Na bismuth iodide in ethyl acetate-glacial acetic acid-water) (136).

The *curare alkaloids* from *Strychnos trinerva* can also be separated into several components, some of which fluoresce, by paper electrophoresis in 25% acetic acid (137). In this class of substances, which contains tertiary and quaternary compounds, paper electrophoresis may be used for separation of very small amounts, since only quaternary ammonium ions migrate to the cathode even in strongly alkaline electrolyte solutions (e.g. in 0.1 *N* NaOH) (138).

L. Inorganic Ions

1. Cations

Following the demonstration by Kendall and White (139) as early as 1924 that Ca^{++} and Ba^{++}, among other ions, were easily separable in a NaCl-containing agar gel, and after the introduction of filter paper as the supporting medium, numerous publications dealing with the separation of inorganic cations of all groups have appeared, principally from the laboratories of M. Lederer (140) and H. H. Strain. A significant feature of this procedure is the fact that electrophoresis of heavy metals must be carried out in the presence of ions which form more or less stable complexes with the cations to be separated, since the free metal ions are strongly adsorbed on the paper. Only a small selection from the ever expanding multitude of publications can be presented here.

The alkali and alkaline earth ions with the exception of rubidium and cesium can be separated very effectively in 0.1 *M* ammonium carbonate solution (pH 8.9) at 100 volts per centimeter under cooling, as shown by Gross (141) and represented schematically in Fig. 12.

Rb and Cs can be separated if a longer migration distance is used, although their ionic mobilities differ only by 1.2%. Ba < Sr < Mg and Ca migrate to the cathode in the stated order in 0.05 *M* citric acid solution adjusted to pH 5.5 with NH_4OH; after 45 minutes they are sharply separated from each other by a distance of 2 cm. each. After evaporation of the volatile $(NH_4)_2CO_3$ from the paper (10 minutes at 80°C.), the alkali metals are detected as the hydroxides with bromothymol blue; the alkaline earths are visualized by spraying with alizarin S and exposure to NH_3 vapor. Other cation separations are also indicated there.

The metals of the Cu group (Cu^{++}, Cd^{++}, Hg^{++}, Sb^{+++}, Bi^{+++}), for example, may be separated conveniently, according to Strain (142), by

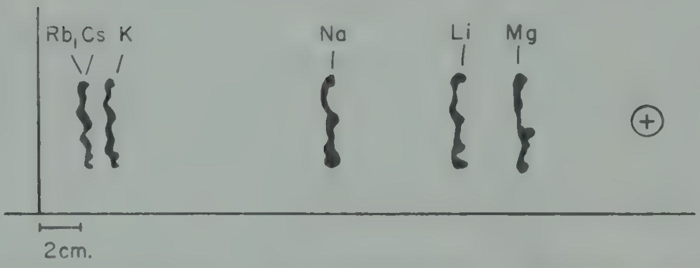


FIG. 12. Paper electrophoresis of alkali metal ions. Ammonium carbonate solution at pH 8.9. [According to Gross (141).]

two-dimensional paper electrophoresis in the manner shown in Fig. 13, *a* and *b* and detected with H_2S or ammonium sulfide. The current is conducted in by simple contact of the platinum wire electrodes with the buffer-moistened paper which is placed between two glass plates. Such an arrangement may also be used for the continuous separation of ions, but the latter is more conveniently carried out in the apparatus constructed for curtain electrophoresis.

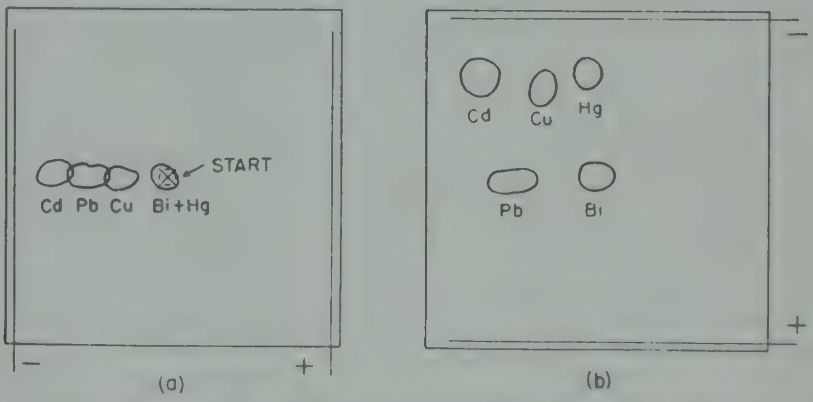


FIG. 13. Two-dimensional pherogram of the metals of the Cu group. (a) In 0.1 *M* lactic acid (first phase); (b) in aqueous NH_4OH (second phase). [According to Strain (142).]

The application of continuous processes may be illustrated by the separation of mixtures of yttrium and lanthanum, lanthanum and actinium. Separation is accomplished with citric acid as the electrolyte and the detection of the separation trails, by spraying with 8-hydroxyquinoline in ultraviolet light (143). Further electrophoretic separations of complicated mixtures of *rare earths* have also been possible in citric acid (144). Concerning the paper electrophoretic separation of *precious metals*, see recent publication (145), to give one example. Paper electrophoresis is particularly suitable for the detection of small traces of isotopic metals, e.g. Sr^{90} (1).

2. Anions

The anions of inorganic acids differ greatly in their mobility in the electrical field and can therefore be separated particularly well, for instance the halides in lactic acid (146) or ClO_2^-, ClO_3^-, and ClO^- in triethanolamine-acetic acid at pH 8.4 (147). This method has also served well in the examination of seleno- or tellurothionates for uniformity (148). Their application to the field of phosphates was discussed in Chapter II.

The great mobility of most of the ions encountered in biochemical experiments (K^+, Na^+, Ca^{++}, Mg^{++}, Cl^-, SO_4^{--}, HPO_4^{--}) permits their removal, i.e. the *desalting* of small amounts of material by electrophoresis on filter paper or filter pasteboards (149). This is of value for all analytical processes in which larger amounts of inorganic salts interfere.

In addition to the reviews cited under (140) and (149), mention may be made of (150–152).

ACKNOWLEDGMENT

The assistance of Dr. Elfriede Niemann in the selection of this material and in the preparation of the bibliography is gratefully acknowledged.

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